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Method Developments To Analyze Caffeine And Acesulfame-K Concentrations In Natural Waters In And Around Charleston, Il, Usa

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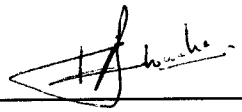

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Method Developments to Analyze Caffeine and Acesulfame-K Concentrations in Natural Waters in and around Charleston, IL USA

(TITLE)

BY

Laleen Bodhipaksha

THESIS

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IN THE GRADUATE SCHOOL, EASTERN ILLINOIS UNIVERSITY
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Method Developments to Analyze Caffeine and Acesulfame-K Concentrations in Natural Waters in and around Charleston, IL, USA.

Laleen C. Bodhipaksha

I would like to dedicate this thesis to

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Abstract

Analytical methods using batch solid phase extraction were developed for two chemical markers, caffeine and acesulfame-K, to assess anthropogenic water contamination of natural water bodies in and around Charleston, Illinois.

Measurements were conducted on 12 different extracted samples using reverse phase HPLC. Two creek samples showed the presence of acesulfame-K (0.45 ± 0.10 and 0.25 ± 0.06 ppb) and two creek samples showed the presence of caffeine (3.1 ± 1.2 and 1.6 ± 0.6 ppb). Effluent from the Charleston waste water treatment plant showed an acesulfame-K concentration of 0.67 ± 0.15 ppb.

These results indicate measurement of both markers is helpful in identifying contamination sources in natural water bodies.

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1. Introduction

1.1. Water pollution due to pharmaceuticals, personal care products and food industry additives

The pollution of water due to pharmaceuticals, personal care products (PPCPs) and food industry additives has been a major problem as they can cause known and suspected adverse ecological and human health effects. The consumption of PPCPs has been increasing very rapidly since 1994. This is confirmed by the increasing expenditures for the production of PPCPs by 75 % from 1994 to 2000[1]. The increase in use of these PPCPs leads to increase in the amount of medication that enter into and pollute natural water bodies. According to the U.S. EPA Office of Research and Development's Strategy Plan 2000, PPCPs are considered to be one of the top five dominant contaminants that affect human and ecological health[2].

The presence of pharmaceutical chemicals in natural water bodies was first recognized in the mid 1970's[3]. The 2002 report from the U.S Geological Survey has found that U.S. waterways contain medications, such as Acetaminophen (0.009 μg/L), tylosin (0.050 μg/L), codeine (0.100 μg/L), carbodox (0.100 μg/L), Ibuprofen (0.018 μg/L)[4].

The presence of personal care products such as shampoos, toothpaste, insect repellents, deodorants and perfumes in natural waters has been recorded. Tonalide and galaxolide are synthetic musks, which are common ingredients in fragrances for cleaning products. Bruchet, et al. found the presence of these two chemicals in Seine River water, downstream of Paris at less than 0.005 μg/L in concentration[5]. N,N-diethyl-meta-

toluamide, (DEET) and dimethyl phthalate are used in insect repellents. Gregory, et al. found that the presence of these two chemicals in raw drinking water at 0.010 and 0.148 μg/L in concentration respectively. Also, the presence of dimethyl phthalate in finished drinking water was found to be at 0.040 μg/L in concentration. Surfynol, a non-ionic surfactant which is used in internal can coatings, paper coatings, etc, has been found at 0.158 and 0.043 μg/L in concentration in raw and finished drinking water respectively. Triclosan, used in toothpaste, was detected at 0.056 and 0.049 μg/L in concentration and Bis(2-ethylhexyl)phthalate (DEHP), a plasticizer which is used in manufacturing of articles made of PVC, was detected at 0.66 and 0.34 μg/L in raw and finished drinking water respectively. Diethyl phthalate, a plasticizer and used in fragrances and cosmetics and benzophenone, used in plastic products, have been detected in raw and finished drinking water at μg/L levels in concentration[6].

The use of different types of food additives to increase the sweet taste of foodstuffs also has been increasing. For ages, natural carbohydrates or additives produced from them were used for making foods delightful to eat[7]. But as the demand for sweet low-calorie food increased the use of artificial intense sweeteners has grown. At present eight high intense non-nutritive sweeteners such as acesulfame-K (ACS-K), aspartame (ASP), aspartame-acesulfame salt, cyclamate (CYC), saccharin (SAC) sucralose (SCL), neohesperidine dihydrochalcone (NHDC) and thaumatin have been included in European Union legislation for use in foodstuffs. Out of the above sweeteners ACS-K, ASP, CYC, SAC, SCL and NHDC are artificial compounds[8]. Butylated hydroxyanisole (BHA), a widely used food additive, has been found in the presence of raw and finished drinking water at 0.54 and 0.23 μg/L in concentration respectively[6].

It is worth to discuss how these PPCPs and food industry additive get into water. Basically there are four different theoretical ways that would bring these materials into natural water bodies[9].

Manufacturing facilities: Materials or compounds that are used for producing pharmaceutical or other products may be discharged with wastewater from the factory.

Consumer discards into treated wastewater: Unused, partially used, expired or old medications may be discarded form houses, hospitals, pharmacies and veterinary practices into the wastewater. Also detergents, shampoos and insect repellents etc are discarded into wastewater.

Excretions into treated wastewater: Drugs and their metabolites and also bioactive compounds like caffeine and nicotine metabolites are excreted by the consumers. So these materials can reach the wastewater treatment plant through the sewer system from houses, schools, hospitals, business places etc. In fact, humans do not absorb 50-90% of active ingredients in drugs thus they are excreted[10].

Discards and excretions into runoff flowing to water bodies or groundwater: Discarded and excreted substances may be carried in the runoff from private septic tanks, manure or sewage sludge spread in the farm lands, treatment facilities for livestock and aquaculture operations and landfills etc. All these methods, however, bring the pollutant to the natural water streams. These contaminations may lead to severe environmental problems. The presence of alien substances in the natural waters may affect aquatic life including fish and plants, birds and other animals that use aquatic plants and fish as their food sources and humans if they use contaminated water for drinking.

There are several documented examples of PPCP's creating environmental problems. Diclofenac, is being used as a painkiller in cattle[11]. Wide spread uncontrolled use of this drug in the Indian subcontinent has been toxic to vulture populations due to contamination of excess chemicals[12]. It has been found that ingestion of this drug harms vultures' kidneys and kills the birds in days. Estrogen compounds have been found in the natural waters in significant concentrations[13]. Intake of these chemicals by male fish causes feminization[14]. The wide spread use of antibiotics results in significant concentrations in surface waters which could make alterations in the microbial population and affect the food chain. Prozac and Paxil are used to treat depression and Luvox is used to treat obsessive-compulsive disorders in humans. Fong, P. has found the presence of these chemicals in natural water samples and his experiment has shown that these three substances induce spawning in zebra mussels even at very low concentrations[15]. This could potentially lead to a rapid increase of bivalve population which in turn may interfere with water intake pipes used for electric generating plants, other industries and municipal drinking water plants. The eastern US has been facing this problem and a colossal amount of money has been spent on cleaning and controlling projects[16]. Some PPCPs, for instance nitro and amino-nitro musks, have been found to be very high acute toxins which could lead to subtle ecological changes[2].

The half life of these compounds sometimes can be long or short. But it is not necessarily needed to be too long if they are continuously adding to the natural water bodies. The concentrations of pollutants are as mentioned elsewhere at parts per trillion or parts per billion levels. The exposure of these aquatic pollutants can be

multigenerational. So even though each small change is undetectable, long term irreversible changes can result[2].

The concentration levels of these pollutants in natural water samples are low and variable. A German researcher, Heberer, T., measured the maximum concentration of 270 ng/L for clofibric acid which is a metabolite of the cholesterol controlling drug clofibrate in humans in Berlin tap water[17,18]. In the following table (Table 1) the results from comprehensive testing of pharmaceuticals, known or potential endocrine disrupter compounds and other wastewater contaminants in source water, finished drinking water and tap water from nineteen U.S drinking water treatment plants collected during 2006-2007 has been listed[18].

1.1.a. The utility of tracers

In order to assess the presence of contamination in natural water samples, chemical markers are used. When selecting a marker the following criteria should be taken into account: high consumption, occurrence in natural waters and/or waste waters, limited biodegradation and availability of analytical methods[5]. Caffeine and acesulfame-K have been utilized in the tracer analysis for many cases.

1.1.b. Chemistry and fate of caffeine in the environment

Caffeine is known as one of the most widely used substances in the world and still the demand in a variety of products is increasing daily. It can be found in beverages, foods, tobacco, condiments and medication. Researchers have found that the

Table 1: Concentrations of the pollutant in the water sample in source water, finished drinking water and tap water from nineteen U.S drinking water treatment plants collected during 2006-2007 [18]. All concentration values are presented in ng/L.

	Source		Finished		Tap Water	
Pharmaceuticals	**Maximum**	**Median**	**Maximum**	**Median**	**Maximum**	**Median**
Atenolol	36	2.3	18	1.2	0.84	0.47
Atorvastatin	1.4	0.8				
Carbamazepine	51	4.1	18	6	10	6.8
Diazepam	0.47	0.43	0.33	0.33		
Diclofenac	1.2	1.1				
Fluoxetine	3	0.8	0.82	0.71	0.64	0.64
Gemfibrozil	2.4	2.2	2.1	0.48	1.2	0.43
o-hydroxy atorvastatin	1.2	0.7				
p-hydroxy atorvastatin	2	1				
Meprobamate	73	8.2	42	5.7	40	5.2
Naproxen	32	0.9				
Known or potential EDCs						
Atrazine	870	32	870	49	930	50
17-estradiol 0	17	17				
Estrone	0.9	0.3				
17R-ethynylestradiol	1.4	1.4				
Bisphenol	14	6.1	25	25		
Butylbenzyl phthalate	54	53				
Diethylhexyl phthalate	170	150				
Galaxolide	48	3	33	31		
Linuron	9.3	4.1	6.2	6.1	110	
Nonylphenol	130	1000	100	93		97
Progesterone	3.1	2.2	0.57	0.57		
Testosterone	1.2	1.1				
Other chemicals						
BHT	49	49	26	26		
Metolachlor	81	17	27	16	22	18
DEET	110	85	93	63	63	49
TCEP	530	120	470	120	200	150
TCPP	720	180	510	210	240	220

average consumption of caffeine per person per day is 70 mg but it can vary from country to country[19]. A pot of coffee generally contains about 1500 to 1800 mL of coffee. Therefore households that consume coffee could produce a significant amount of caffeine daily[20]. Tang-Liu found that after the ingestion of caffeine, it is extensively metabolized in the human body and 3% of it is excreted unmetabolized with urine[21]. Thus one can argue that households discharge only very small amounts of caffeine into waste water. But in reality large amounts of caffeine are added to the domestic waste water from the disposal of unconsumed coffee, tea, soft drinks through household drainages[20]. Caffeine concentration determinations in Miami River and Mississippi River have been found to be 0.0218-0.0412 μg/L[22] and 0.01-0.07 μg/L[19]. The study of the concentrations in sewage effluent have been measured at > 100 μg/L in the USA, 20-300 μg/L in Canada and 34 μg/L in Sweden. In the outflows of sewage treatment plants in Texas the detected caffeine concentration was 1.3-2.4 μg/L and in groundwater wells underneath a landfill in Barcelona it has been detected at 0.04-0.23 μg/L[19]. These experimental data showed that caffeine is present in natural water samples in detectable concentrations.

Figure 1 shows the structure of caffeine (1,3,7-trimethylxanthine),

Information on the fate of caffeine in the environment is very limited. Studies have shown that after release of caffeine into waste water, it slowly under goes degradation and is transformed to variety of xanthine products[23]. It has been revealed that *Pseudomonas* and *Aspergillus* mainly contribute to this degradation

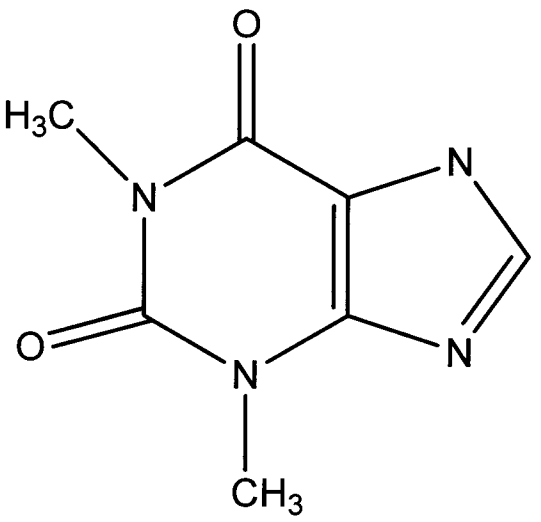


Figure 1: The structure of caffeine

process[23,24]. Bacteria degrade this compound via demethylation but the mechanism of degradation by fungi is still unknown[24]. Ralph, L. S. found the presence of chlorinated derivatives of caffeine in waste water samples. Since caffeine is not conserved (it degrades) they have suggested that use of caffeine as a tracer is limited[20]. But further consideration on available information about caffeine is noteworthy. The high solubility (13.5 g/L[22] in water), low octanol water coefficient (log Kow = 0.01)[25] and insignificant volatility of caffeine make it more likely to remain in water rather than with organic molecules such as humic materials[22]. Therefore most researches consider caffeine as a good marker for the determination of the presence of PPCPs and food additives in the natural water bodies. In fact, it is a good tracer for anthropogenic influences on natural waters.

1.1.c. Chemistry and fate of acesulfame-K in the environment

Acesulfame-K, 6-methyl-1,2,3-oxathiazine-4(3*H*)-one-2,2-dioxide potassium salt, is a synthetic substance that is used in various food products as a sweetener worldwide. Figure 2 shows the chemical structure of acesulfame-K and it is a sulfoamide.

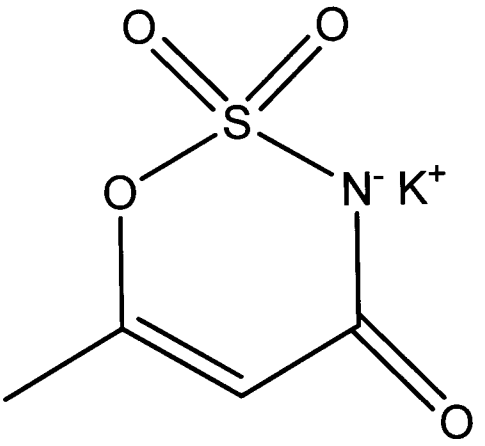


Figure 2: The structure of acesulfame-K

After ingestion of this compound with foodstuff, most of it is not affected by human metabolism. Thus a large part of this is excreted through urine and feces[26]. Therefore there is a good chance of this chemical to reach the environment via domestic waste water. Buerge et al, found acesulfame-K concentrations at 12-46 μg/L in treated and untreated wastewater samples, up to 2.6 μg/L in several tap water samples and up to 4.7 μg/L in ground water samples from Switzerland[26]. These data shows that this substance is present in the environment in detectable concentrations. Further, experimental data has revealed that the global consumption of acesulfame-K in 2005 was to be 4000 tonnes[26] which confirms it is released into the environment in significant quantities.

At the ambient pH values of natural waters acesulfame-K is ionized and behaves like an anionic compound[26]. Humic substances that are present in water also bear negative charges due to carboxylic and phenolic groups. Because of this reason acesulfame-K is most likely to dissolve in water rather than stick with organic substances.

1.2. Techniques for analysis of pharmaceuticals, personal care products and food industry additives in water samples

Researchers have been using different types of analytical techniques to determine the house hold waste water contamination in natural water bodies for ages. Generally, PPCPs and food industry additives are present in natural waters in very low concentrations such as at ppb (μg/L) levels. Thus, either the analytical technique that is expected to be used for contaminant concentration analysis should capable of measuring very low concentrations or one should use a concentrating process prior to do the analysis. Let's discuss some analytical techniques that are used for tracer analysis.

1.2.a. Enzyme-linked immunosorbent assay (ELISA)

Basically the purpose of ELISA is to determine the presence of a particular antibody or antigen in a sample and quantify it[27]. It is relatively a cheap and sensitive technique[28].

The solid phase used in ELISA may contain cross-linked dextran or polyacrylamide beads, filter paper or polypropylene tubes and polystyrene microtitre plates. The specific antigen or antibody is attached to this solid phase by passive adsorption or covalent interactions[28]. The antigen bound solid phase is washed to avoid non-specific interactions or hydrophobic bonding which could lead to incorrect results.

There are two main analytical ELISA techniques: the competitive method and the double antibody method. In the competitive method, a mixture of known amount of enzyme labeled antigen and an unknown amount of unlabelled antigen is reacted with a specific antibody adsorbed to solid phase. Then this complex is washed with a buffer and enzyme substrate is added. After that, the enzyme activity is measured. In the mean time, the enzyme activity of a complex that has not been mixed with unlabelled antigen is measured. The difference these two values is the concentration of the unlabelled antigen[28].

In the double antibody method first unknown antigen is reacted with specific antibody attached to solid phase. Then it was washed followed by reacted with enzyme labeled antibody. After it has been washed, enzyme substrate is added. Finally, the enzyme activity is measured under standard condition. This value is directly proportional to the concentration of unknown antigen[28].

1.2.b. Chromatographic method

For these types of tracer analyses researchers use chromatographic techniques such as Gas Chromatography coupled with Mass Spectrometry (GC/MS), High Performance Liquid chromatography (HPLC) and HPLC/MS. In GC/MS technique a mass spectrometer acts as the detector and gas chromatography separates the components of a mixture[29,30]. By combining of these two techniques, researchers can use it for both quantitative and qualitative analysis of solutions containing many chemicals. In GC the mobile phase is an inert gas such as He[29]. Once the sample is injected to the mobile phase separation is begun. This mobile phase carries the sample mixture to a stationary

phase which is usually a column that selectively interacts with the components in the injected sample mixture. Components that have more affinity with the stationary phase elute from the column later whereas the components that have lower affinity towards the column elute first. After the elution, components enter a detector which creates electronic signals once the presence of a component is detected. These signals are then processed by a computer program and the chromatogram of intensity (abundance) vs retention time is generated. If the GC is connected with a MS, then the eluted components enter the mass spectrometer. Then they are bombarded by a stream of electrons to generate fragments. Most likely these fragments possess a +1 charge. After detection the mass spectrum is created by a computer program. In this spectrum y-axis represent the abundance data and x-axis represent the mass to charge ratio (M/Z) values. A mass spectrum of a particular compound is considered as its fingerprint. So it can be used to identify unknown materials[29]. Therefore, for tracer analysis research work GC/MS has been very valuable. For instance, if we are interested to check the presence of a particular marker in the water samples GC/MS analysis can be utilized.

In HPLC, the mobile phase is a liquid. In reverse phase chromatography a non-polar stationary phase such as C-18 is used with a polar mobile phase. As in the GC, injected sample mixture is carried to the stationery phase. Depending on the affinity of the components in the sample mixture, elution takes place. As the elution occurs they enter a UV-detector and generate signals by measuring absorptions. The higher the concentration of the component, the bigger the absorbance. These signals are then processed by a computer program and chromatogram which shows intensity in Y-axis and retention time in X-axis is generated. Each component has a unique retention time for

a given set of chromatographic conditions. By changing the characteristic of the mobile and stationary phase separation of components can be adjusted.

When HPLC is utilized in tracer analysis, first a suitable method is generated to separate the compound of interest. Using this method, retention time for a solution of this compound is measured. If the unknown sample contains this compound, its chromatogram should show a peak at this retention time. To determine the concentration of the unknown sample, the peak integrations for known and unknown samples are taken into account. If the HPLC is combined with MS, as the compounds elute from the HPLC column, they enter mass spectrometer. Then the identification of the components can be carried out as mentioned in the above GC/MS analysis.

1.2.c. Other techniques

Gibbons, et al., used capillary electrophoresis combined with UV detection (CE/UV) for determination of pharmaceutical and personal care products in wastewater[31]. They were able to reach the detection range of 1.6 - 68.7 ppb via solid phase extraction. They have found this technique to be low cost, simple, fast and fairly sensitive method for the analysis of various PPCPs in wastewater samples. The capillary electrophoresis basically contains four different components such as two buffer reservoirs, very small diameter capillary tube, high voltage power supply and a detection system[32]. In this technique the migration of ions in solution under influence of an electric field is taken into account[33]. Different species or solutes have different mobilities in the presence of an electric field; therefore they migrate through the capillary at different speeds. The difference of charge to mass ratio of different ions causes for the difference in rates of

migration. Positively charged species moves towards the negatively charged electrode and vice versa for the negatively charged ions[32]. After separation of ions, the mass spectrometric detection is used for quantitative and qualitative determinations with the electrospray interface[33].

1.2.d. Caffeine and acesulfame-K specific procedures from the literature

Literature data indicate that the concentrations of caffeine and acesulfame-K in surface water are at μg/L levels. So prior to qualitative or quantitative analyses there should be a concentration process. Buerge, et al., used a solid phase extraction (SPE) process for this purpose[26,34]. The extraction was done with reusable columns containing approximately 10 mL of a macroporous polystyrene adsorbent (Bio-Beads SM-2, 20-50 mesh, Bio-Rad Laboratories, Hercules, CA). They used separate adsorbent columns for waste water treatment plant samples and surface water samples in order to prevent cross-contamination. Before the columns had been used they were conditioned with dichloromethane, methanol, and fossil groundwater. For both caffeine and acesulfame extractions, 1 L of solution was passed through the SPE column at a rate of 10 mL/min. Elution was done using methanol and dichloromethane. The two eluents were combined and shaken vigorously to get the layers separated. After that dichloromethane layer were evaporated under gentle draft of air. For acesulfame-K the residue was reconstituted using 5 mL of water and it was analyzed using HPLC-MS technique. For HPLC the used method was linear gradient from 95% 1mM ammonium acetate, 5% methanol to 100% methanol within 20 min followed by isocratic phase of 5 min and 0.2 mL/ min flow rate. For caffeine, the concentrated sample in dichloromethane was analyzed by GC/MS.

Carvalho, J.J developed a new immunoassay based on a commercially available anti-caffeine monoclonal antibody and a newly synthesized tracer, using horseradish peroxidase and UV–visible detection[35]. The achieved limit of detection was 0.001 μg/L and the solid phase extraction was found to enhance the detection limit. The concentrations above 0.025 μg/L could be quantified with a relative error lower than 20%.

1.3. Embarras River Watershed

The Embarras River is a 314 km long tributary of the Wabash River in southeastern Illinois in the United States. It rises in the Champaign County, IL and eventually water of the Embarras River reaches the Gulf of Mexico through the Wabash, Ohio and Mississippi Rivers. The Embarras River passes all the way through the towns of Villa Grove, Camargo, Charleston, Greenup, Newton, Ste. Marie and Lawrenceville and the counties of Champaign, Clark, Coles, Crawford, Cumberland, Douglas, Jasper, Lawrence, and Richland[36,37]. Our study was carried out in the Coles county (Charleston) area. Several creeks in the Charleston area connect with the Embarras. And out flows of the treatment plants of Charleston and Mattoon are connected to it.

The Embarras River is nominated as a Biologically Significant Stream. The broad sand and gravel substrate of the middle part of the river make a territory for a number of rare species, such as the harlequin darter, eastern sand darter, bigeye shiner, and blue sucker. And also the diversity of Mussel is found to be considerably high[37].

Figure 3: Map of the Embarras River
Ref:http://upload.wikimedia.org/wikipedia/commons/2/2a/Embarrasrivermap.png (accessed on May 31, 2011)

1.4. Solid Phase Extraction of caffeine and acesulfame-K

For detection of very low concentrations of contaminants, concentrating techniques, such as a column or batch methods should be used. C-18 solid phase extraction columns are a common choice. Another option is the use of a reusable macroporous polystyrene-divinyl-benzene absorbent such as Bio-Beads. This adsorbent is comprised of a large amount of highly cross-linked microspheres which gives this

compound high surface area and uniform pore sizes. These are non-polar and useful for the adsorption of non-polar substances or surface active species in aqueous solutions. Bio-Bead adsorbents have been used to separate water soluble steroids, phenols, drugs, pesticides, trace organics and rhodamine[38]. Bio-Beads can be used with variety of solvents like alcohols, petroleum ether, diethyl ether, hexane and with solvent mixtures and also with aqueous media and it can be utilized in the pH range of 2-10 without damaging it.

1.5. Scope and goal of this work

The overall goal of this research work is to develop a long-term trace chemical measurement program to help assess the exposure and potential vulnerability of the Lower Embarras River watershed to endocrine disrupter contamination. In this project basically two markers, caffeine and acesulfame-K, were used to determine the domestic contamination on the natural water bodies. One question is whether released treated waste water brings domestic waste materials into the river system. Also, are there significant additional inputs (e.g., domestic septic tanks) to the watershed? Finally, which marker, caffeine or acesulfame-K, is the appropriate one for answering these questions in a small town environment?

2. Materials and Methods

2.1. Equipment

(a) For absorbance determination a Shimadzu UV-160 UV-Vis spectrophotometer was used.

(b) An Hitachi HPLC with L-7100 Pump, D-7000 Interface, L-7200 Autosampler, L-7300 Column Oven, L-7420 UV-Vis Detector was used to analyze for caffeine and acesulfame-K. Analysis of both compounds was performed with a C-18 column at a temperature of 30 °C and injection volume utilized was 10 μL. Used mobile phases and UV detection wavelengths will be stated under experimental methods in different cases. For both analyses isocratic methods were used.

(c) A GC/MS with Hewlett-Packard 5890 Series II gas chromatograph with a HP 5971 series mass selective detector and a HP-5MS capillary column with an inner diameter of 0.25 mm was utilized. The system was used in single ion mode for caffeine analysis, monitoring charge to mass ratios of 194.1 and 109 in the low resolution mode. The injector temperature was set to 200 °C and the detector temperature to 280 °C. A splitless injection, with a 1.5 minute purge delay, was used to inject the sample. The initial oven temperature was 90 °C, held for 4.0 min, then ramped at 30 °C/min to a final temperature of 230 °C, which then was held for 10 minutes.

2.2. Chemicals

Acesulfame-K (purity ≥ 99.0%), HPLC grade, purchased form Fluka Analytical, Germany and caffeine, HPLC grade, were used to prepare the standard caffeine and acesulfame-K solution samples. Acetonitrile, methanol (0.2 micron filtered HPLC grade) and O-phosphoric acid 85% from Fisher Scientific, New Jersey were used to make mobile phase solutions. In order to prepare these mobile phase and standard solution samples 10 MΩ cm filtered millipore water was used. Same brand and quality of methanol solution was used for the elution and sample reconstitution processes of caffeine. Nitrogen gas compressed tanks were purchased from Gano Welding Supply, Charleston, IL and it was used in the sample evaporation processes. Biotechnology grade Bio-Beads, SM-2 adsorbent, 20-50 mesh from Bio-Rad laboratories was used for concentrating processes.

2.3. Solid Phase Extraction and chromatographic methods

For the concentration process solid phase extraction methods were used. Initially, the process was begun with use of a C-18 cartridge and eventually Bio-Beads were utilized for this purpose.

2.3. i. Selection of a suitable sorbent and concentration process for caffeine

2.3.i.a. Caffeine extraction by C-18 cartridge

First, 50 ppb caffeine standard sample was prepared using millipore water. Then 1 L of this solution was passed through a C-18 column at a rate of 10 mL/min. After that,

elution was carried out with 10 mL methanol. This eluted sample was evaporated to dryness under nitrogen in a hood. The residue was reconstituted using 2 mL of methanol. This sample was tested with HPLC. For this analysis 100 % methanol was used as the mobile phase and detection was done at the UV wavelength of 274 nm.

The sample also was analyzed with GC/MS using helium gas mobile phase and a HP-5MS capillary column with an inner diameter of 0.25 mm.

2.3. i.b. Caffeine extraction by Bio-Beads column

Caffeine solution (50 ppb) was prepared using millipore water. A Bio-Beads column was prepared by packing 25 g of it in a glass column. Columns were pretreated by flowing methanol through each column prior to extraction. 1 L of the caffeine solution was passed through the column at a rate of 10 mL/min. Elution was carried out using 10 mL of methanol passing through the column. The eluted sample was evaporated to dryness under nitrogen in a hood. The left over after the evaporation process was dissolved in 2 mL of methanol and the sample was tested with HPLC. For this analysis also 100 % methanol was used as the mobile phase and the detection was done at the UV wave length of 274 nm.

A 1L of pond water sample was spiked with caffeine to make a 1 ppm caffeine solution. Then it was filtered and column extraction was done as mentioned above. After the evaporation process the reconstituted sample was filtered using 0.2 μm nylon filters. This sample was tested with HPLC.

Then non-spiked pond water sample was tested for caffeine using column extraction. For that a filtered 1L of pond water sample was used for column extraction.

2.3.i.c Caffeine extraction by Bio-Beads batch method

First 50, 25, 15, 5 ppb caffeine standard solutions were prepared using millipore water and a 10 ppm caffeine stock solution. 500 mL of each standard solution was stirred with 20 g of Bio-Beads for 12 hrs. After that it was filtered using vacuum filtration and for elution the separated Bio-Beads was stirred with 50 mL of methanol for 6 hrs. Then it was filtered and the filtrate was kept under nitrogen in a hood for evaporation to dryness. Once the evaporation was complete the residue was reconstituted with 1 mL of methanol. This sample was tested with HPLC. For the HPLC analysis 10 % acetonitrile and 90 % millipore water was used as the mobile phase and the absorption was detected at UV wavelengths of 210 nm and 274 nm.

Before starting each extraction process, cleaning of Bio-Beads was done by stirring twice with 50 mL of methanol for 3 hrs followed by stirring twice with 50 mL of millipore water for 1 hr.

2.3.ii Acesulfame-K method development

2.3. ii.a. A detection wave length for acesulfame-K

Different pH solutions (~1, 3, 5, and 7) of 10 ppm acesulfame were prepared using millipore water, 6 M sodium hydroxide and 6 M hydrochloric acid. Then UV

absorptions from 200 to 800 nm were measured for each using UV absorption spectrometer.

The absorption of 0.2 M phosphoric acid solution, adjusted to pH ~7 with NaOH, was also taken from 200 to 800 nm using the UV spectrometer.

2.3.ii.b Acesulfame-K extraction by Bio-Beads batch method

In order to prepare the acesulfame-K standard solutions, first, 10 ppm acesulfame-K solution was prepared. For that 13.3 mL of 15M phosphoric acid was added into a 1 L volumetric flask. The required amount of acesulfame-K powder was added to it and millipore water was added up to calibration mark. From this stock solution 50, 20 and 5 ppb acesulfame-K solutions were prepared. Then 500 mL of each solution was stirred with ~20 g of Bio-Beads and the pH of the solution was set up to 2 using 6 M sodium hydroxide and stirred for 12 hrs. Then it was filtered using vacuum filtration to get the Bio-Beads separated. For the elution process the Bio-Beads were stirred with millipore water at pH ~7 for 6 hrs. In this case also 6 M sodium hydroxide solution was used to make the required pH of the solution. After the elution, mixture was filtered by vacuum filtration then the filtrate was evaporated to dryness under nitrogen in a hood. The residue was reconstituted with 1 mL of millipore water and this solution was tested with HPLC. UV detection was done at the wave length of 227 nm and 90 % 0.2 M phosphoric acid and 10 % methanol adjusted to pH ~7 with NaOH was used as the mobile phase.

Before starting each extraction process, cleaning of Bio-Beads was done by stirring twice with 50 mL of millipore water for 3 hrs.

For method development with a natural water sample, a 50 ppb spiked pond water sample was prepared. Then it was filtered and the above mentioned extraction and elution processes were carried out. After the evaporation process the reconstituted sample was filtered using 0.2 μm nylon filters. This sample was tested with HPLC.

2.3. iii. Analysis of water sample from creeks and out flow from the waste water treatment plant

2.3.iii.a. Sampler and sampling process

When collecting water samples in order to reach the water level of the creek, a sampler created using PVC tubes and a bottle was used. It has two lines of tubes, which are possible to be lengthened by attaching tubes, connected to the neck and the bottom part of the sampling bottle. The length of the sampler was adjusted according to the depth to the water level from the sampling point. Glass bottles were used to collect water samples for caffeine analysis whereas plastic bottles were used for acesulfame-K. In order to prevent from any breakage, the glass bottles were covered with a layer of Duct Tape. Bottles with 500 mL volume were used and from each location 1 L of water was taken into the laboratory.

Table 2 contains data on sampling location, sampling date and sampling time.

Table 2: Water sample collected locations, Time and dates.

Sample NO (SN)	Sampling Location		Sampling Date	Sampling Time
	Longitude	Latitude		
1	39.49° N	- 88.18° W	5/4/2011	1.30 p.m
2	39.41° N	- 88.18° W	5/9/2011	2.30 p.m
3	39.49° N	- 88.11° W	5/12/2011	2.00 p.m
4	39.44° N	- 88.17° W	5/14/2011	11.00 a.m
5	39.50° N	- 88.16° W	5/16/2011	11.30 a.m
6	39.49° N	- 88.21° W	5/18/2011	2.30 p.m
7	39.39° N	- 88.16° W	5/23/2011	10.30 a.m
8	39.50° N	- 88.20° W	5/25/2011	10.15 a.m
9	39.47° N	- 88.22° W	6/1/2011	10.30 a.m
10	39.46° N	- 88.19° W	6/6/2011	11.00 a.m
11	39.50° N	- 88.21° W	6/14/2011	10.00 a.m
12	39.44° N	- 88.17° W	6/16/2011	2.00 p.m

2.3. iii.b. Test for caffeine

First, 500 mL of collected water sample was filtered and then it was stirred with 20 g of Bio-Beads over 12 hrs. After that, the mixture was filtered by vacuum filtration to get the Bio-Beads separated. Then for the elution, Bio-Beads were stirred with 50 mL of methanol for 6 hrs. Mixture was filtered using vacuum filtration then the filtrate was kept under nitrogen for evaporation to dryness in a hood. Residue was dissolved in 1 mL of methanol and it was filtered using a 0.2 μm nylon filters before it had been put into HPLC sample vial. This solution was tested with HPLC using the conditions mentioned in part 2.3.i.c. The above procedure was repeated in each case to analyze collected water samples to test for caffeine.

2.3. iii.c. Test for acesulfame-k

First, 13.3 mL of 15 M phosphoric acid was put into a 1 L volumetric flask and then a water sample was poured into it up to the calibration mark. It was filtered and 500 mL of this solution was stirred with 20 g of Bio-Beads. While stirring, the pH of the solution was set up to ~2. Then the stirring was continued for another 12hrs. After that it was filtered by vacuum filtration and elution was done by stirring the separated Bio-Beads with millipore water at pH ~7. Mixture was filtered using vacuum filtration and filtrate was evaporated to dryness under nitrogen in a hood. Residue was reconstituted with 1 mL of millipore water. Then it was filtered using 0.2 μm nylon filters and poured into a HPLC sample vial. This solution was tested for acesulfame-K with HPLC using the method stated in the section 2.3.ii.b.

3. Results and Discussion

3.1. Selection of a suitable sorbent and a concentration process for caffeine

In order to determine the presence of caffeine and acesulfame-K as markers of anthropogenic influences on natural water bodies, primarily a good concentrating process should be utilized as discussed in the introduction. For this purpose extraction and elution processes were tested using different types of solid phase extraction techniques. The basic idea of this concentrating process is to get adsorbed all the substances of interest in a large volume of water sample into an adsorbent material and take the adsorbed substances out from it. Initially, cartridges with C-18 absorbent material were tested in the extraction process with solution of very low concentrations of caffeine. The obtained HPLC chromatogram for the 50 ppb caffeine solution is shown in Figure 4. The obtained retention time and the peak area for this analysis are 4.67 min and 77306 units, respectively. After the HPLC analysis the same sample was tested with GC/MS in order to confirm the presence of caffeine in the sample. The obtained GC/MS chromatographic results are shown in Figure 5. The peak at 12.321 min in the total ion chromatogram confirms the presence of caffeine in the tested sample. The mass spectrum of the above peak further confirms the presence of caffeine. So these all observations prove that C-18 columns can be used to extract caffeine in a solution. Although it worked well as a sorbent the presence of unwanted peaks in the HPLC chromatogram (see Figure 4) needs to be considered. Further analysis regarding this showed that those impurities had been added to the sample during the extraction process which means contaminants probably had come from the C-18 material which contains long chain of organic compounds. If the

testing sample was containing these compounds in it there would be a good possibility to have some peaks around 2 - 3 min retention time. This is because methanol (100 %) had been used as the mobile phase for this HPLC analysis earlier elution of these organic compounds would take place. The appearance of these unwanted peaks in the chromatogram would cause some difficulties in the future when these methods are being utilized to analyze natural water sample. Therefore a different type of a sorbent material was decided to be tested to use in the extraction process.

Buerge, et al. used columns containing a macroporous polystyrene adsorbent (Bio-Beads SM-2, 20-50 mesh) material to their solid phase extraction procedures of extraction of caffeine[34]. This sorbent material helped them to obtain cleaner extracts than compared to that of the C-18 silica sorbents.

Using the Bio-Beads column, a 50 ppb caffeine standard solution was extracted and the obtained result is showed in Figure 6. The recorded retention time for the analysis was 4.71 min and the peak area was 39142 units. Since the mobile phase used for this HPLC analysis is same as in the previous analyses, the retention times for the elution of the substance of interest should be either equal or very close. The experimentally obtained values are close enough to be consistent with this. The Figure 4 shows a higher peak area than that of the Figure 6, which suggest that C-18 is a more effective adsorbent than Bio-Beads. However, in both cases it was difficult to control the solution flow rate through the column material which influenced reproducibility. This might be the major reason for the peak area difference. But a good improvement was observed in the use of Bio-Beads, that is, it was able to get rid of unwanted peaks around 2-3 min in the chromatogram. Considering these results and observations it was decided to use Bio-

Beads in the future extraction process, in fact, further studies of use of Bio-Beads were carried out.

A solution of 1 ppm caffeine spiked pond water sample was tested with concentration process and HPLC analysis in order to understand the feasibility of utilization of found method in analysis of natural water samples. Figure 7 showed the obtained chromatogram for this experiment. According to the figure it is possible to understand that the obtained retention time for this analysis is consistent with that of standard samples. This proved that the method used worked in the analysis of natural water samples. Further analysis of the identification of unwanted peaks in the Figure 7 shows that these are due to presence of some compounds such as dissolved biological and unknown substances in the natural water sample. But the presence of these substances do not disturb to our analysis because of having a longer retention time for the elution of caffeine. Since the found method was showing an improvement a blank (non-spiked) pond water sample was analyzed. Figure 8 shows the chromatogram of this work. The results show some signal at the relevant retention time, so if caffeine were to be present in the sample at a low concentration it would be difficult to identify in the chromatogram. This is likely due to the difficulty in cleaning the Bio-Beads on the column.

A more efficient concentrating process needed to be utilized to reach the detection limit of the spectrometer. Therefore experimenting for a newer extraction method was done. Besides getting the Bio-Beads clean, a major weak point of the previous column extraction methods was the difficulty in the maintaining a slow, steady and consistent flow rate of solution through the column. In order to avoid this, batch method extraction was taken in to account.

3.2. Optimization of the procedures for caffeine analysis

When the 100 % methanol was used as the HPLC mobile phase the retention time for the caffeine elution was around 4.7 min. If Figure 7 is studied well it can be seen that the pre-elution of other dissolved substances in the natural water sample may perturb the absorption peak that appears due to the elution of caffeine. Therefore if the retention time of the elution of caffeine could be increased it will be much easier to have a very clear absorption peak. This can be done in various ways such as adjusting the mixing ratio of the mobile phase, changing the pH in either the mobile phase or the testing sample, putting a longer HPLC column, changing the flow rate etc. For this investigation the adjusting of mobile phase was studied. Since the caffeine is readily soluble in methanol, with the mobile phase of methanol the elution of caffeine would occur soon. By the study of solubility, caffeine is found to be less soluble in water than in methanol at room temperature. Therefore this time 90 % water and 10 % acetonitrile was tested as the mobile phase in the HPLC that gave the retention time around 15.5 min for the elution of caffeine.

Different concentrations of standard caffeine solutions such as 50, 25, 15 and 5 ppb were analyzed using HPLC after they had been extracted from the batch method. UV absorptions in the HPLC analysis of these extracted samples were tested in two different wave lengths; those were at 210 and 274 nm, in order to choose a better wave length for the detection. Figure 9 (a), (b), (c) and (d) show the HPLC chromatogram results obtained by detecting at 210 nm UV wave length where as figure 10 (a), (b), (c) and (d) show the chromatogram results which were taken by detecting at 274 nm.

Since the concentrations of the substance in the test solution to be analyzed is at ppb levels, it is very important to detect the absorption at the optimum wave length. In order to understand this, for instance, Figure 9 (c) and 10 (c) can be compared. The same sample of extracted 15 ppb caffeine solution has been tested at these two wavelengths. The chromatogram of figure 10 (c) shows a high intensity peak for caffeine that was detected at UV wave length of 274 nm. This is consistent with the analysis for other concentrations too. Therefore UV wave length of 274 nm was decided to be used in the future analysis of caffeine. At this stage some modifications were done in the elution process also. Previously, it was done by just passing 10 mL of methanol through the Bio-Beads by which caffeine had been adsorbed. Figure 6 shows an example of HPLC analysis for this extraction method. Eventually, it was started to use the batch method for elution step as well. The HPLC chromatogram of Figure 10 (a) is from a sample which was prepared by the use of both method for both extraction and elution process. This both tests have been done for 50 ppb caffeine standard solutions. The peak area value which shows the concentration of the caffeine is bigger in Figure 10 (a) than in Figure 6. This suggests that the efficiency of the recovery of the substance of interest is much higher when the batch method is used in the both processes of the concentration step.

3.3. A detection wave length for acesulfame-K

It was very essential to find out a suitable UV wave length in order detect acesulfame-K in the HPLC analysis. Figure 11 shows the absorption spectra of 10 ppm acesulfame solutions at pH 7 scanned from 200 to 800 nm. A buffer solution was required to be used in the mobile phase of the HPLC in the analysis acesulfame-K.

Because of that the above study was carried out to understand the variation of absorption intensities as varying the pH of the solutions. This study shows that the maximum intensity of the absorption takes place at 227 nm for every different pH of acesulfame-K solutions. Therefore the UV wave length of 227 nm was used in the future analysis of this compound.

3.4. Finding a SPE and a chromatographic methods for analysis of acesulfame-K

In natural water acesulfame-K is a salt as discussed in the introduction. Because of that to get it adsorbed to Bio-Beads it should be converted into neutral species. Therefore the extraction was done at pH ~2 (the pKa of acesulfame)[39] to achieve partial neutralization. All the acesulfame-K samples were prepared in 0.2 M phosphoric acid solutions adjusted to pH 2 with NaOH. (The pKa values of phosphoric acid are 2.14, 7.19 and 12.15[40], so using it for a pH 2 buffered extraction solution was appropriate.) The elution process was carried out at pH~7 (again, adjusted via NaOH) where acesulfame is a salt so comes out of the sorbent material easily.

Yang, D.J., et al. and Zygler, A., et al. in their work on the simultaneous determination of nonnutritive sweeteners studied the retention behavior of sweeteners in different SPE sorbents. Compound studied included acesulfame-K and analytical techniques included HPLC/ESI-MS and LC/MS respectively. In order to separate the sweeteners gradient elution was used with a mobile phase of methanol, formic acid buffer and acetone (69:24:7, v/v/v) in A and methanol, formic acid buffer and acetone (11:82:7, v/v/v) in B[4,41]. The recorded retention time in the both cases for the elution of acesulfame-K was 3.76 min. Buerge I.J. et al. utilized a gradient elution with the mobile

phases of 1 mM ammonium acetate in A and methanol in B to separate the sweeteners[26]. In the work the recorded retention time for the elution of acesulfame-K is 11.00 min. In our case also it was required to have a somewhat a longer retention time because as mentioned earlier pre-elution of other substances in the natural water samples may disturb the absorption signal of the acesulfame-K elution. But it was sufficient to use an isocratic elution since this work is not to separate sweeteners but to detect the presence of acesulfame-K.

Isocratic elution of acesulfame-K was done using 90 % 0.2 M phosphoric acid and 10 % acetonitrile mobile phase adjusted to pH 7 with NaOH. The obtained retention time for this analysis was around 6.9 min which is really suitable to our work. Since the detection was done by a UV detector the absorption of 0.2 M phosphoric solution adjusted to pH 7 with NaOH was studied to make sure there was no strong absorption peak at 227 nm. Figure 12 does not show an absorption peak that would interfere with the absorption of acesulfame. Acesulfame-K standard solutions with concentration of 50, 20, 5 ppb were analyzed by means of the developed concentrating and chromatographic method. Figure 13 (a), (b), and (c) show the obtained chromatographic data for this analysis. In order to test for the feasibility of the method to use in the analysis of natural water samples a 50 ppb spiked pond water sample was analyzed. Figure 14 shows the HPLC Chromatogram of extracted 50 ppb acesulfame-K spiked pond water sample. This implies that the disturbance from the pre-elution of other dissolved substances in the natural water has been minimum on the absorption peak of the acesulfame-k.

3.5. Linear response of the developed methods and concentration determinations

In order to study the linear behavior of the developed methods for caffeine and acesulfame-K analysis, graphs of peak area vs concentration were prepared. Figure 15 (a) and (b) show the graph of peak area vs concentration of caffeine in millipore water and the graph of peak area vs concentration of acesulfame-K in millipore water respectively. The trend line of the graphs in Figure 15 (a) clearly illustrate the linear behaviour of the analysis method of caffeine. The R-squared value of 0.995 further proves its linear behaviour. According to the Figure 15 (b) the analysis method of acesulfame-K shows a curvature behaviour over the range of used standard concentrations. However, an assumption was made that the method was sufficiently linear over a small concentration range (0-5 ppb) to allow linear analysis.

In order to find the chemical concentration in an unknown sample only one standard measurment was employed. The relevant peak area in the HPLC chromatogram of an unkonwn was compared with the peak area of a 5 ppb standard acesulfame or caffeine solution analyzed during the same analysis session. For this determination, chromatographic data obtained through one instrumental run was used in each case.

3.6. Error analysis

The uncertainty of caffeine concentrations was estimated using the regression line shown in Figure 15 (a). Standard errors for area, slope and intercept were calculated using excel functions. These values were then used to estimate the standard error for each required concentration.

In order to find the uncertainty for acesulfame concentrations, the regression line shown in Figure 15 (b) was made linear by taking ln(x) = X. This linear version was used to determine the uncertainty value for X which in turn was used in a mathematical relationship to estimate standard errors for each concentration.

3.7. Analysis of water sample from creeks and out flow from the waste water treatment plant.

The developed concentration and HPLC methods were used to determine the presence of caffeine and acesulfame-K in natural water samples from creeks around Charleston City area and the out flow of the waste treatment plant of Charleston. Table 02 includes the data of latitude and longitude, time and date that the samples were collected. Initially, sampling locations were selected randomly considering the places where the anthropogenic influences were found to be available. Eventually, by considering the obtained data, sampling was more targeted. Figure 16 shows the map of sampling sites in Charleston City area.

Acesulfame is an ion in water at ambient conditions. Because of that it can adhere to the glass bottles very easily when sampling and so it can be lost in significant quantities. This is the reason to use plastic bottles to collect water samples for testing acesulfame-K as mentioned in the experimental section. But in the case of caffeine, they are neutral species in natural water, so that use of glass bottles would not be problematic.

Water samples collected from eleven different locations were analyzed for caffeine and acesulfame-K. The HPLC chromatogram results of water sample SN 1, SN 4 and SN 8 show the presence of acesulfame-K. The peak areas of unknown samples (Figures 17

(a), 17 (b) and 17 (c) and standard samples (Figures 18 (a), 18 (b) and 18 (c)) were compared, in order to find out the concentration ranges of acesulfame-K in the above water samples. Table 3 shows the obtained concentrations of acesulfame-K in analysed water samples.

The chromatogram results of SN 4 (Figure 19 (a)) and SN 11 (Figure 19 (b)) for caffeine analyses show the presence of caffeine in these water samples. These results were compared with that of the chromatograms of the standard caffeine samples shown in Figure 20 (a) and 20 (b) respectively, in order to find out the caffeine concentration in the unknown water samples. Table 4 contains the concentration data of caffeine for the analyzed water samples.

Table 3: Concentrations of acesulfame-K in water samples and peak areas of unknown and standard samples that are compared with each other (Note: 'not detected' is abbreviated as ND)

Sample No (SN)	Concentration of acesulfame-K/ ppb	Peak area (Unknown)	Peak area (Standard)
01	0.45 ± 0.10	991	11039
02	ND	NA	NA
03	ND	NA	NA
04	0.25 ± 0.06	695	13546
05	ND	ND	ND
06	ND	ND	ND
07	ND	ND	ND
08	0.67 ± 0.15	2286	17017
09	ND	ND	ND
10	ND	ND	ND
11	ND	ND	ND
12	ND	ND	ND

Table 4: Concentrations of caffeine in water samples and peak areas of unknown and standard samples that are compared with each other (Note: 'not detected' is abbreviated as ND)

Sample No (SN)	Concentration of caffeine/ ppb	Peak area (Unknown)	Peak area (Standard)
01	ND	ND	ND
02	ND	ND	ND
03	ND	ND	ND
04	3.1 ± 1.2	6335	10220
05	ND	ND	ND
06	ND	ND	ND
07	ND	ND	ND
08	ND	ND	ND
09	Not Clear	ND	ND
10	ND	ND	ND
11	1.6 ± 0.6	2740	8404
12	ND	ND	ND

The concentrated testing samples of natural water samples in which the substances of interest have not been detected may contain them at a concentration of lower than the range of the detection limit of the method. For instance, before sample 10 (SN 10) was collected, considerable amount of raining (0.16 in) was receiving over the previous day. (Table 5 shows rainfall data over the previous day of the sampling date).This could lead to dilute the contaminant concentration in the creek water which causes to have much lower concentration in extracted sample than the detection limit of the instrument. In addition to that the addition of water of Cassell and Riley to the Kickapoo may lead to dilute the contaminant concentration at the location 10.

Table 5: Rainfall data over the previous day of the sampling date (Note: TOP Trace of Precipitation) Reff: Weather Underground, *http://www.wunderground.com* (accessed on July 1, 2011)

Sample No	Sampling Date	Rainfall Over the Previous Day/ in
1	5/4/2011	0.00
2	5/9/2011	0.00
3	5/12/2011	0.00
4	5/14/2011	TOP
5	5/16/2011	0.18
6	5/18/2011	0.00
7	5/23/2011	TOP
8	5/25/2011	TOP
9	6/1/2011	TOP
10	6/6/2011	0.16
11	6/7/2011	0.00
12	6/8/2011	0.00

The sample no 8 (SN 8) was from the out flow of the Charleston waste water treatment plant. In this sample, acesulfame-K was detected at a concentration of 0.67 ppb where as caffeine was not detected. The treatment plant carries out biological reactions and chlorination of waste water in their purification process. As discussed in the introduction, as far as the two markers are concerned, caffeine is most likely to undergo biological degradation. It may be that is the reason it is not detected in the water sample 8. In order to study the condition of the water in the creek before the discharge of the treatment plant, SN 11 was collected. Analysis of the sample showed the presence of only caffeine in it at a concentration of 1.6 ± 0.6 ppb. This suggests that some amount of caffeine has been added to the natural water by direct disposal, such as run off of the septic tanks out flows etc. In order to confirm the presence of caffeine in this sample SIM GC/MS analysis was done and Figure 21 (a) shows the chromatogram of the analysis.

The chromatogram was compared with the GC/MS chromatogram collected for a standard 5 ppb caffeine sample which is shown in the Figure 21 (b). The analysis of this standard sample showed a peak at 9.3 min in the SIM GC/MS chromatogram and the unknown sample SN 11 also showed this peak confirming the presence of caffeine.

Water sample no 4 (SN 4) has shown the presence of both caffeine and acesulfame-K. By the comparison of peak areas, SN 4 was found to contain acesulfame-K and caffeine at concentrations of 0.25 ± 0.06 and 3.1 ± 1.2 ppb. This sample was collected from Kickapoo Creek a short distsnce above its entrance to the Embarras River. In order to study the changes of contaminant concentrations through time, another water sample (SN 12) was collected again after one month from the same place and it was analysed for acesulfame-K and caffeine. Figure 22 (a) and (b) show the HPLC chromatogram of acesulfame-K and caffeine analyses respectively. But this time according to the chromatogram data neither caffeine nor acesulfame-K was detected. So this tells, depending on the sampling date results can vary.

Figure 23 shows the HPLC chromatogram data for the analysis of caffeine for the water sample no 9 (SN 9). If the chromatogram is studied carefully, it will be possible to see a very small bump right after 15 min. So it is suspected to have some possibility to contain caffeine in it. HPLC analysis for this sample was done after few days of the completion of the evaporation process. Therefore if some decomposition process was taken place during this period, the compound concentration in the testing sample could be lower than the actual.

The acesulfame-K contamination of the Kickapoo Creek (SN 4) probably is due to both waste water effluent and other external inputs. In addition to that the acesulfame-K contamination in location 1 (SN 1) is because of external inputs. The presence of caffeine in Cassell Creek and Kickapoo Creek probably is primarily due to external (non-waste water treatment) inputs. Therefore, it is possible to understand that the contamination of caffeine and acesufame-K of the natural water bodies in Charleston City area occurs through waste water effluent and other external inputs. This also implies that there are contaminants such as PPCPs and food industry additives in the natural water systems around Charleston, IL, and the sources of these contaminants include incomplete degradation in the waste water treatment process and other inputs.

Conclusions

The study of use of caffeine and acesulfame-K as markers in the determination of anthropogenic influences on the natural water bodies shows very interesting results. According to the detected concentrations, caffeine is present in higher values compared to that of acesulfame-K and caffeine's linear analytical characteristics makes it easier to analyze, but the stability of this compound in the environment is a problem. In the out flow of the waste treatment plant only acesulfame-K was detected. But above the discharge of the treatment plant caffeine was detected. It is suspected that due to the biodegradation of caffeine during the treatment process, it did not show up. The detection of acesulfame-K in the out flow of the WWTP confirms that treated waste water brings domestic waste materials into the river system. The detection of caffeine prior to the discharge of WWTP confirms that there are additional inputs of contaminant into the watershed. Finally, in general, analysis of both provides more complete information regarding contaminate inputs into natural waters.

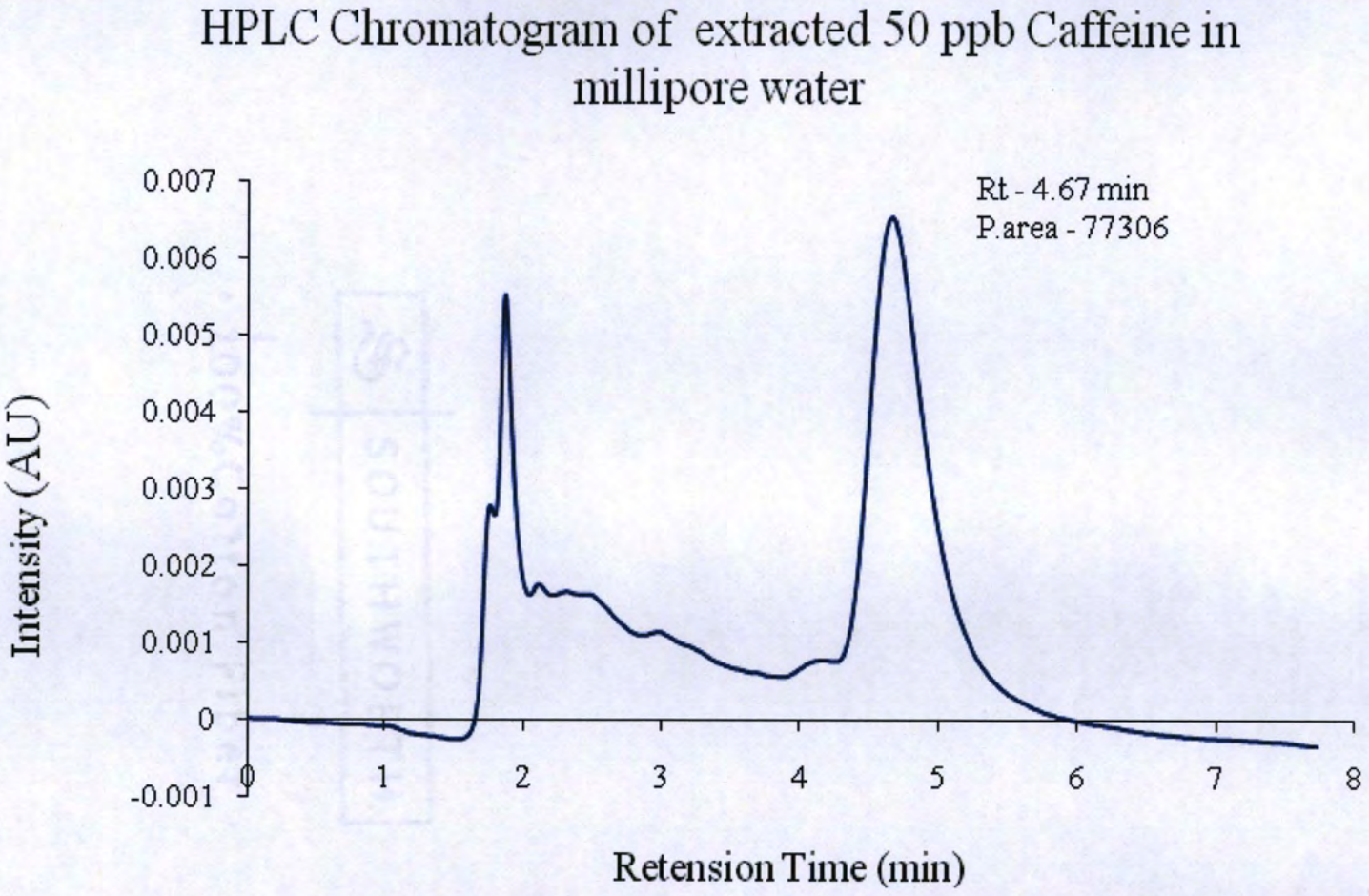


Figure 4: HPLC chromatogram of extracted 50 ppb caffeine in millipore water using C-18 cartridge.

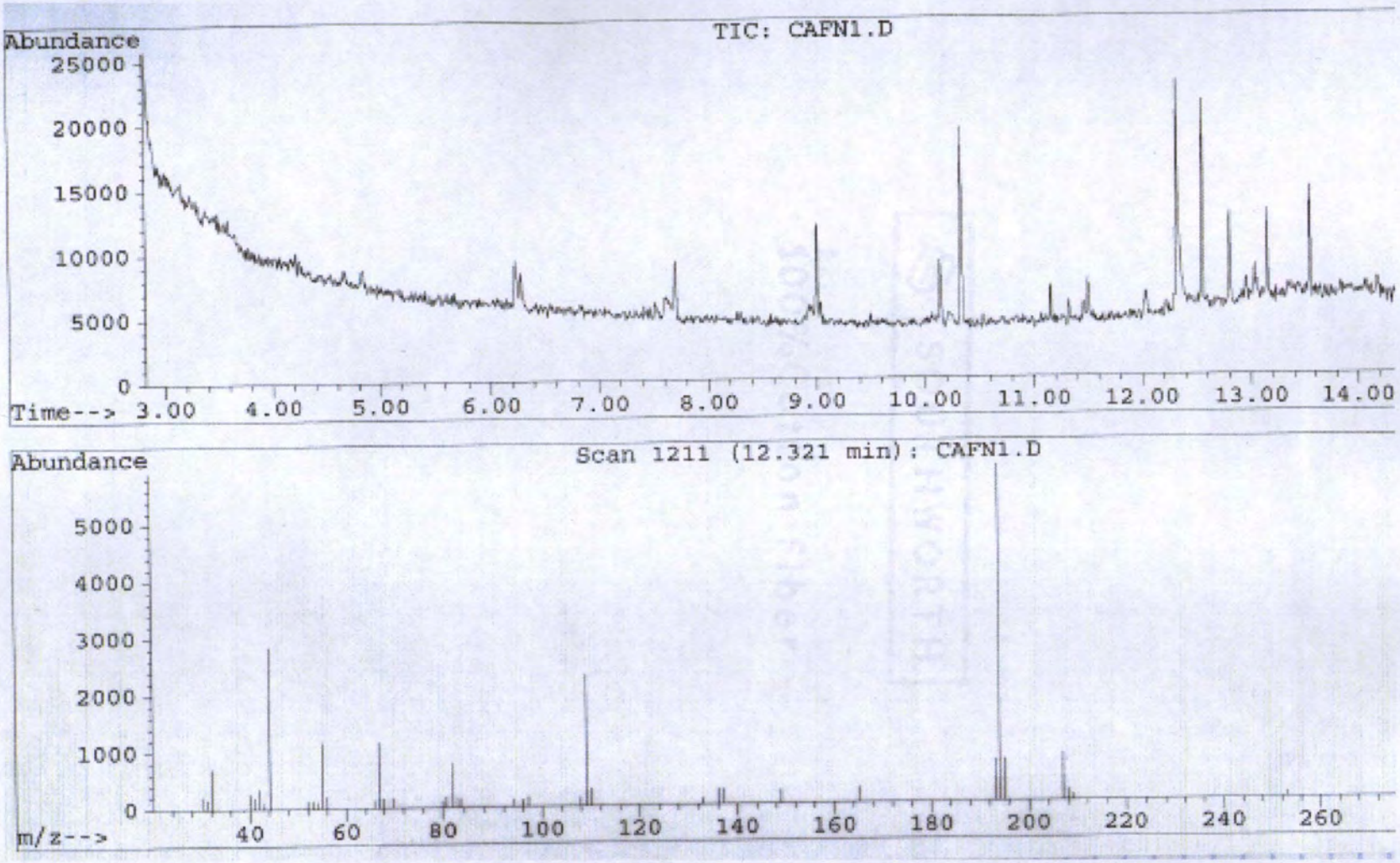


Figure 5: GC/MS chromatogram of extracted 50 ppb caffeine in millipore water using C-18 cartridge.

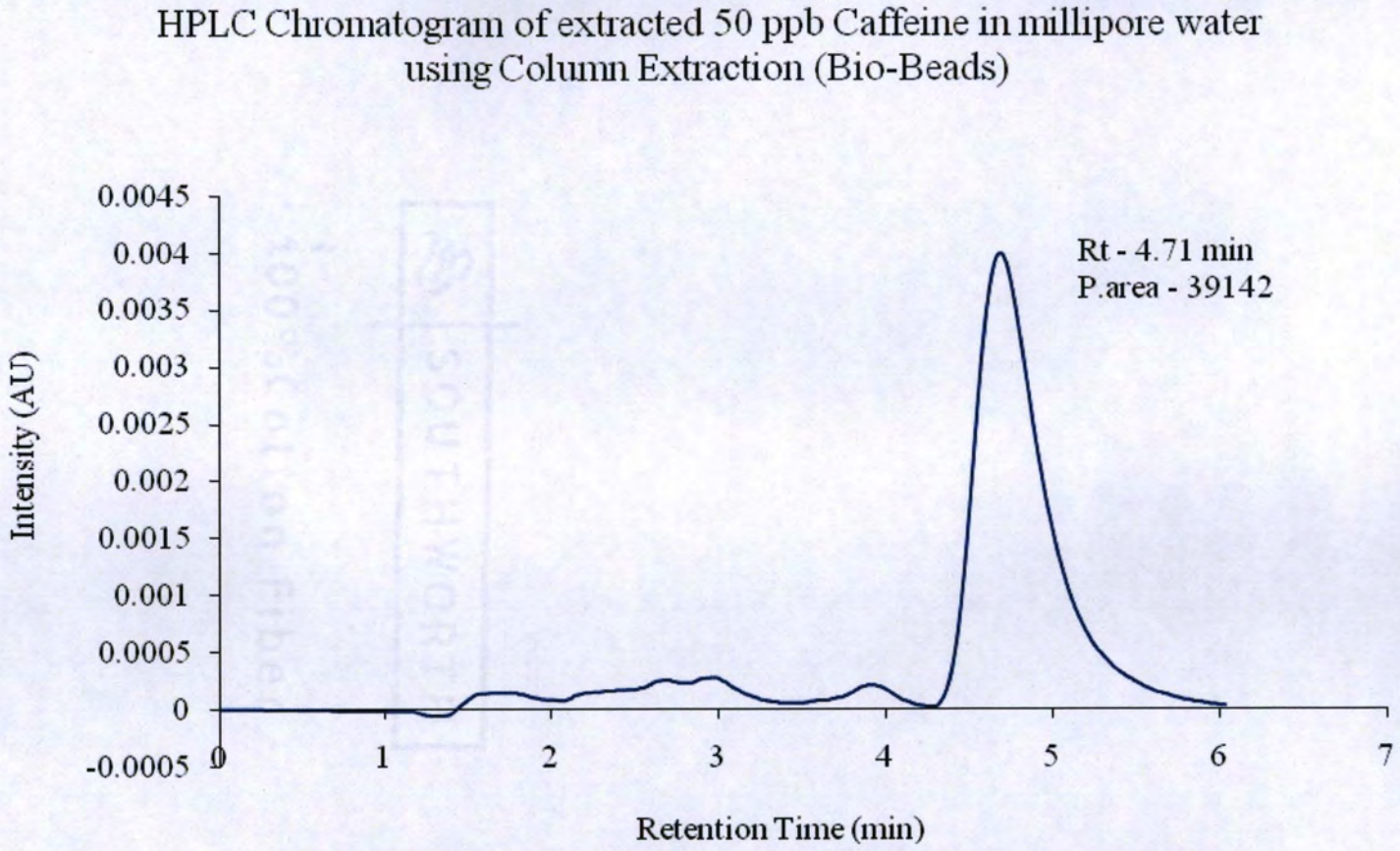


Figure 6: HPLC chromatogram of extracted 50 ppb caffeine in millipore water using Bio-Beads column.

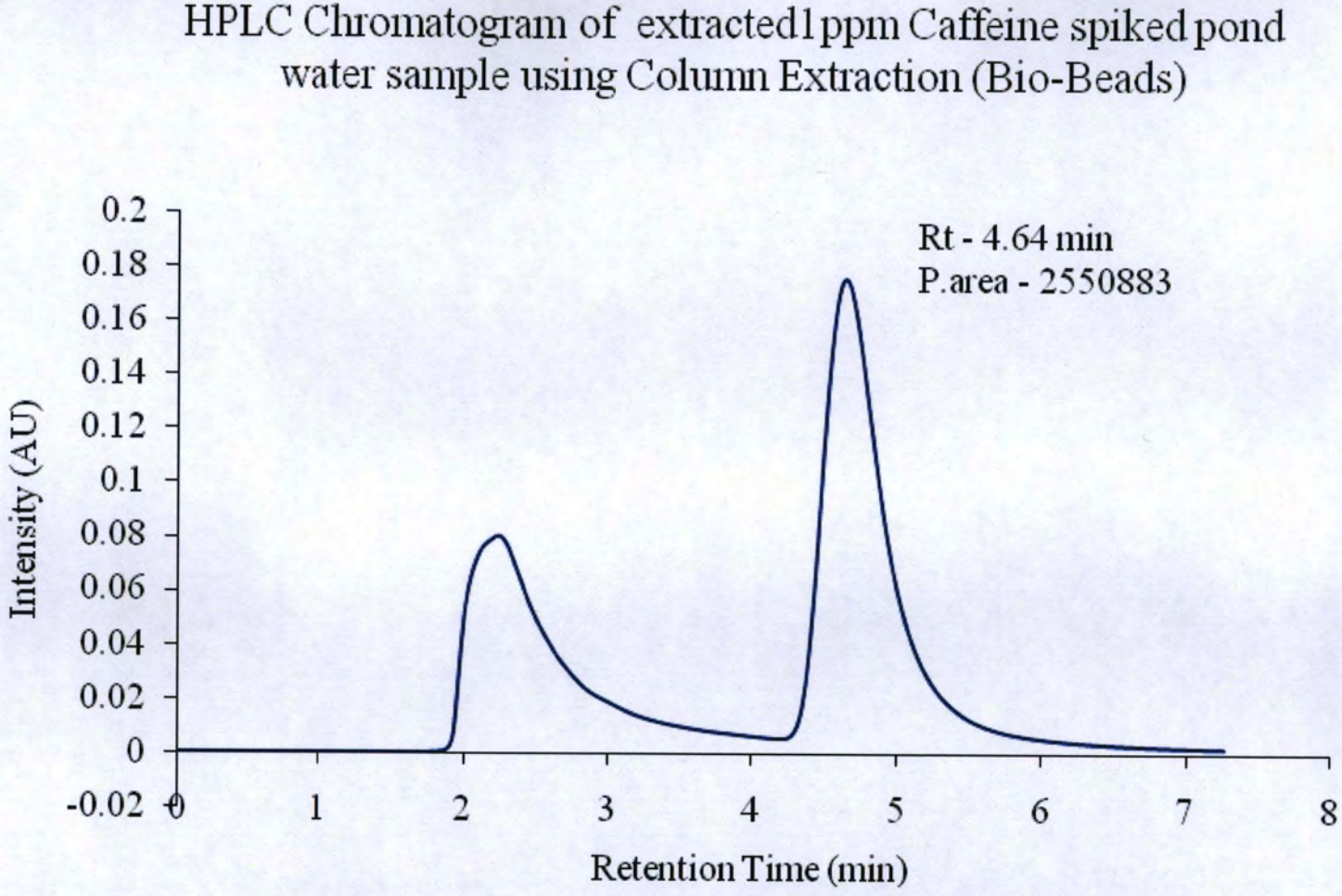


Figure 7: HPLC chromatogram of extracted 1 ppm caffeine spiked pond water sample using Bio-Beads column.

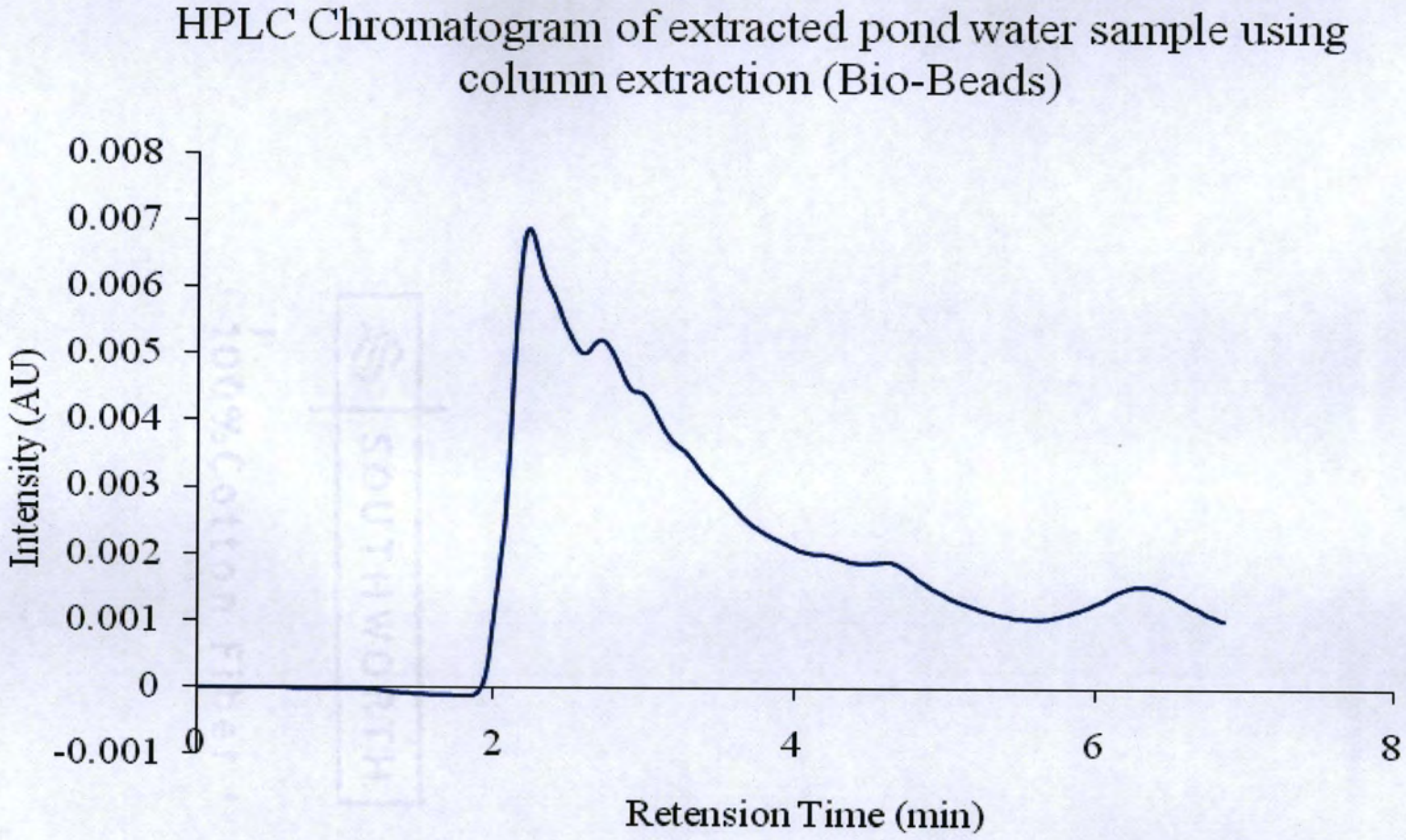


Figure 8: HPLC chromatogram of extracted blank pond water sample using Bio-Beads column.

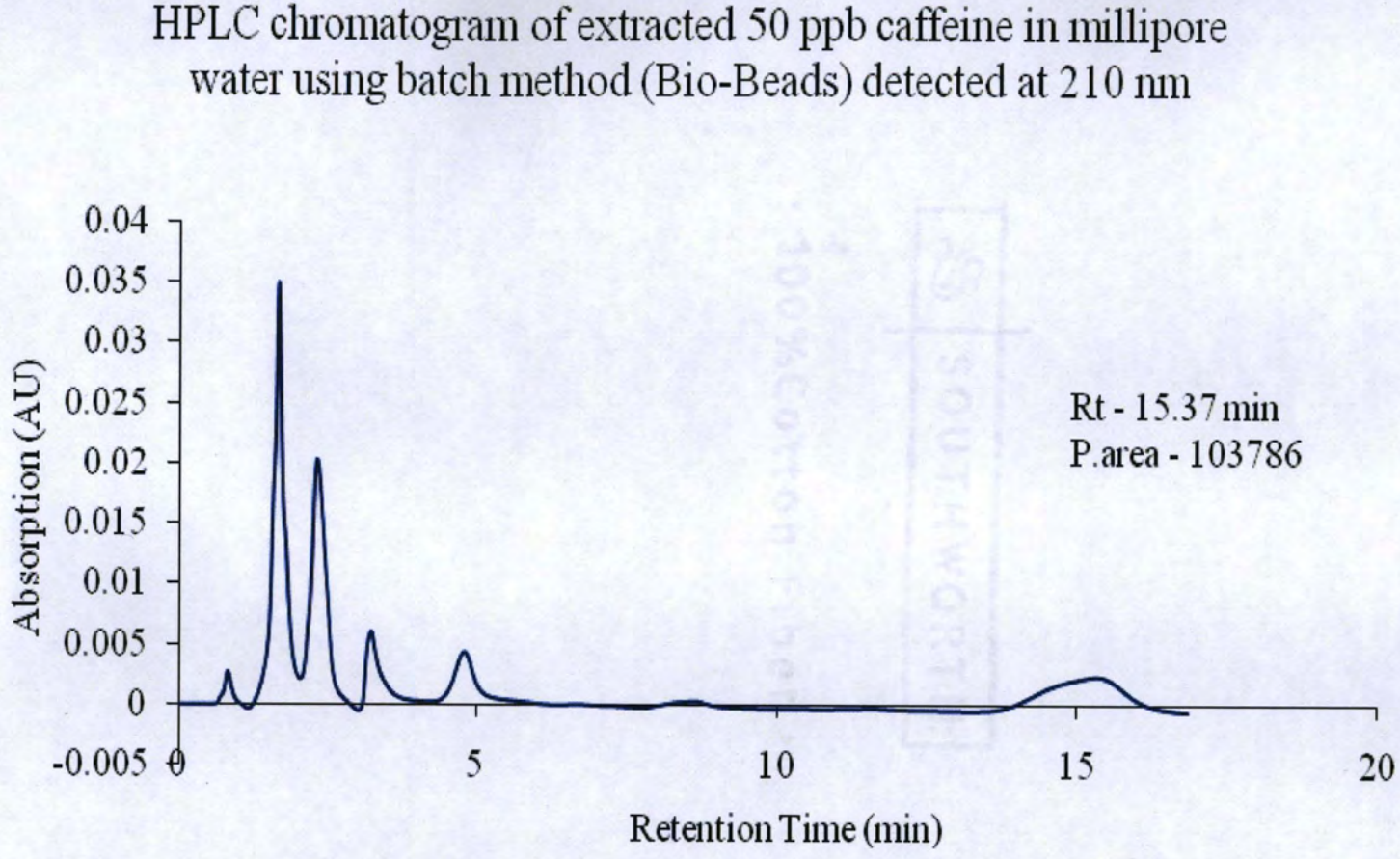


Figure 9 (a): HPLC chromatogram of extracted 50 ppb caffeine in millipore water using Bio-Beads batch method (detected at UV wavelength of 210 nm)

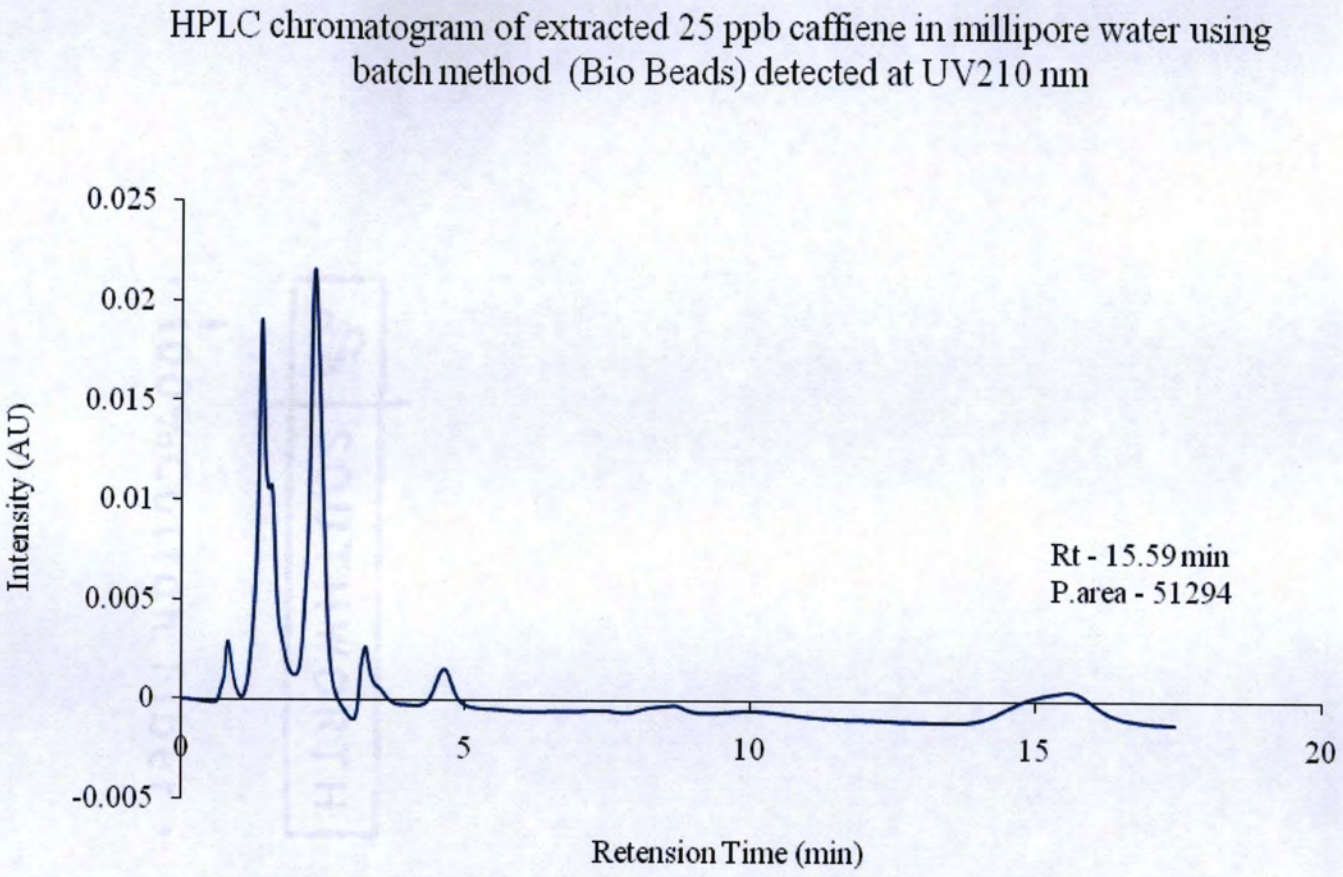


Figure 9 (b): HPLC chromatogram of extracted 25 ppb caffeine in millipore water using Bio-Beads batch method (detected at UV wavelength of 210 nm).

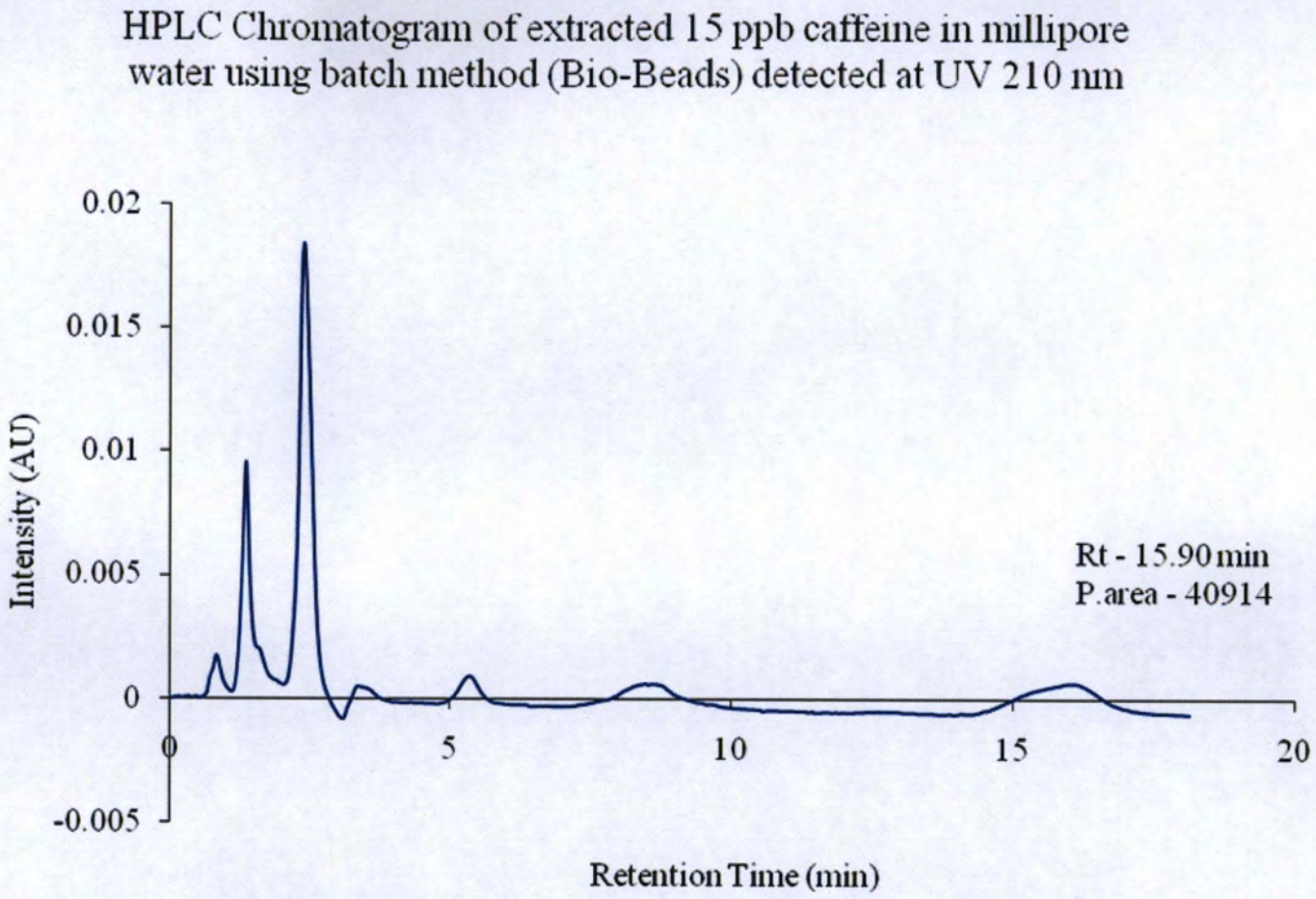


Figure 9 (c): HPLC chromatogram of extracted 15 ppb caffeine in millipore water using Bio-Beads batch method (detected at UV wavelength of 210 nm).

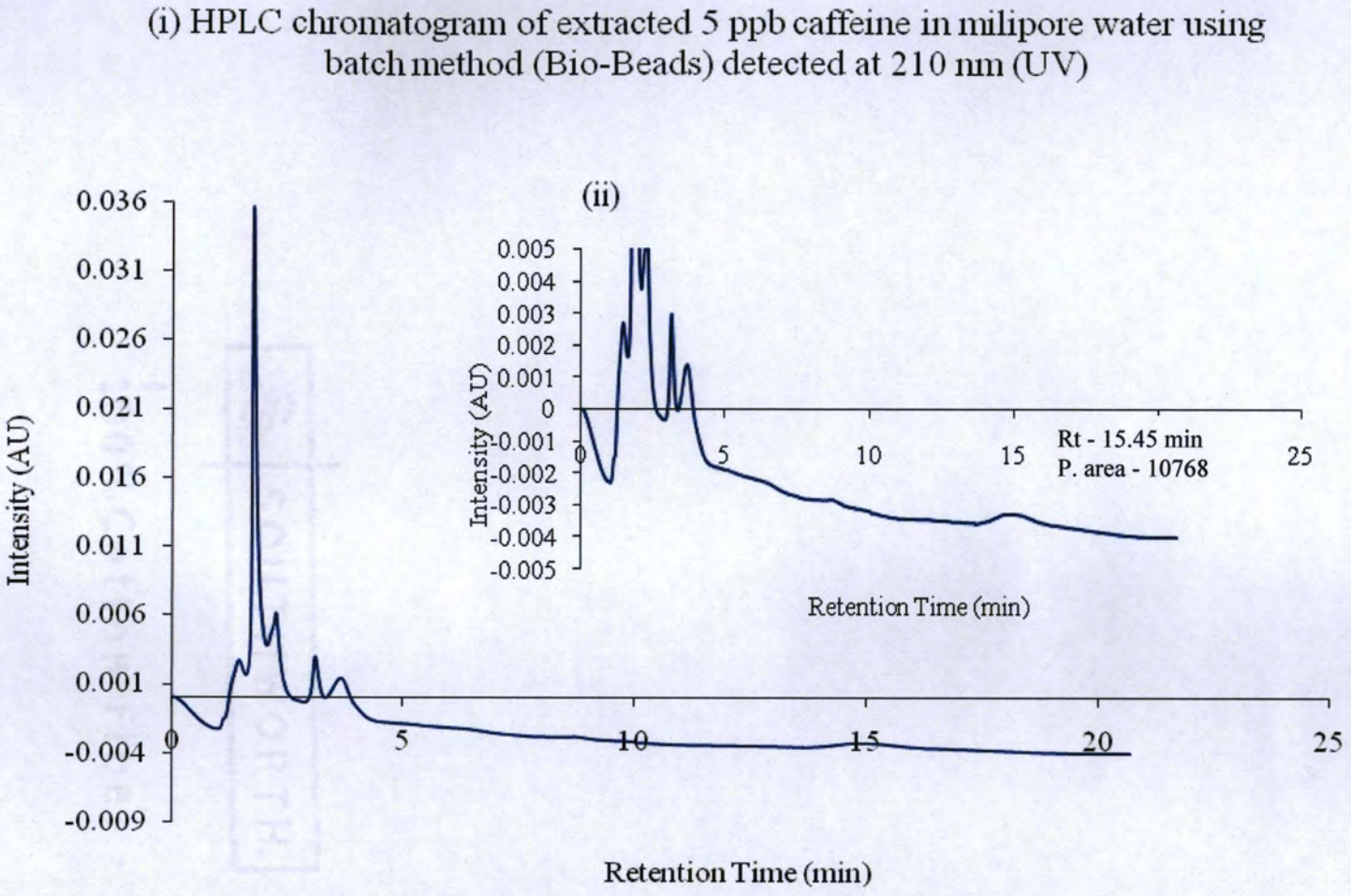


Figure 9 (d – i and ii): HPLC chromatogram of extracted 5 ppb caffeine in millipore water using Bio-Beads batch method (detected at UV wavelength of 210 nm). Note: The graph (i) and (ii) are made in different scales of maximum values in y axis for more clarity.

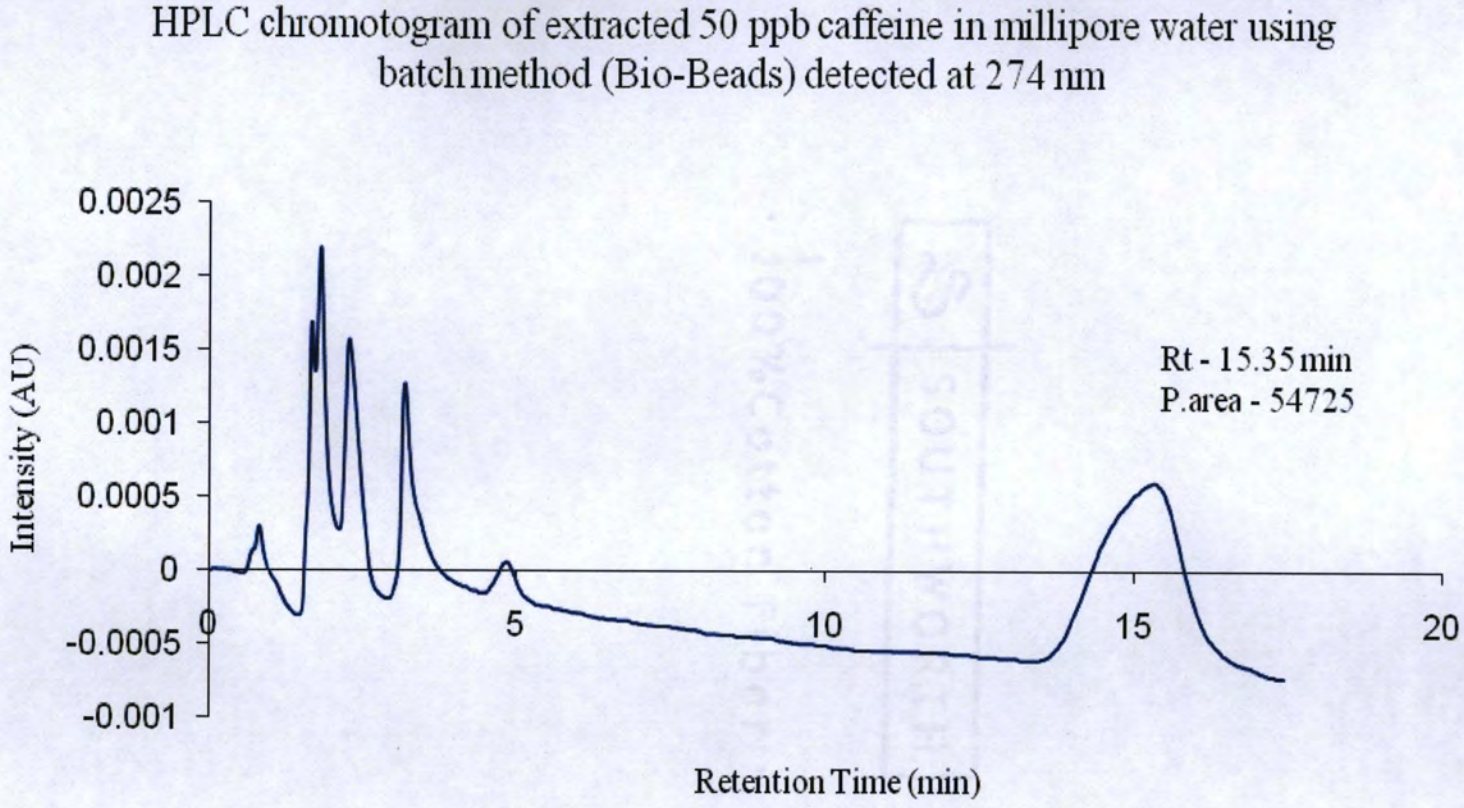


Figure 10 (a): HPLC chromatogram of extracted 50 ppb caffeine in millipore water using Bio-Beads batch method (detected at UV wavelength of 274 nm).

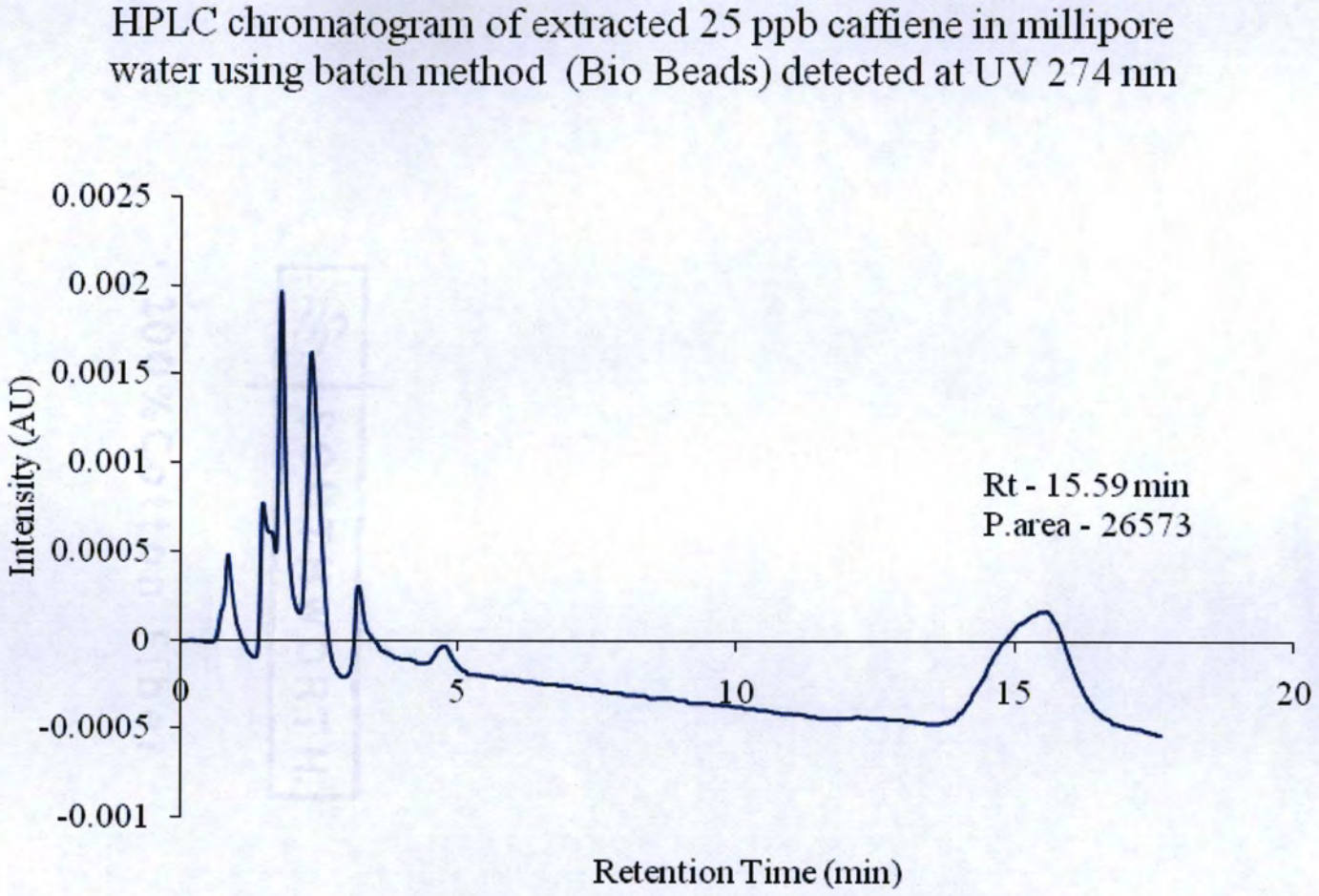


Figure 10 (b): HPLC chromatogram of extracted 25 ppb caffeine in millipore water using Bio-Beads batch method (detected at UV wavelength of 274 nm).

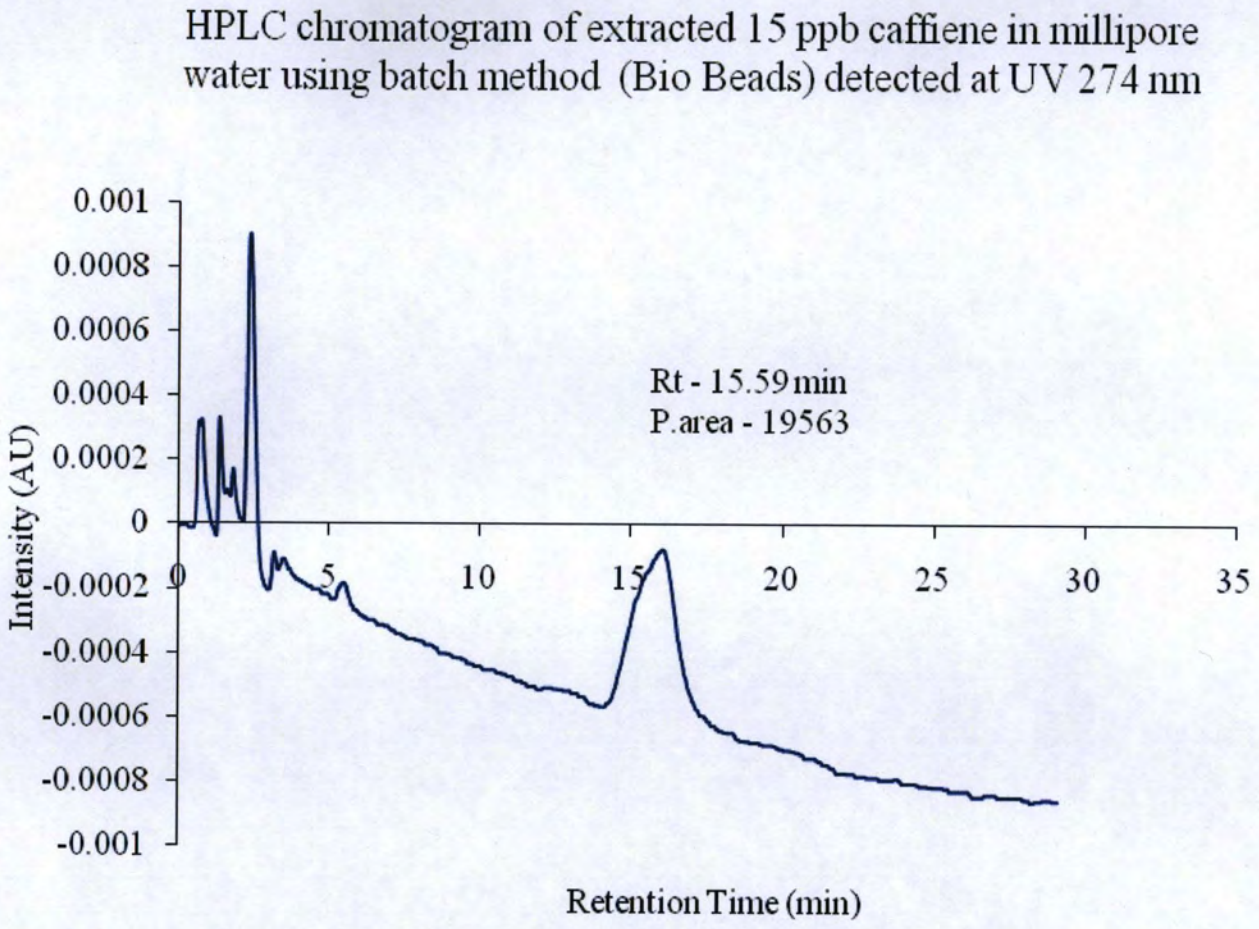


Figure 10 (c): HPLC chromatogram of extracted 15 ppb caffeine in millipore water using Bio-Beads batch method (detected at UV wavelength of 274 nm).

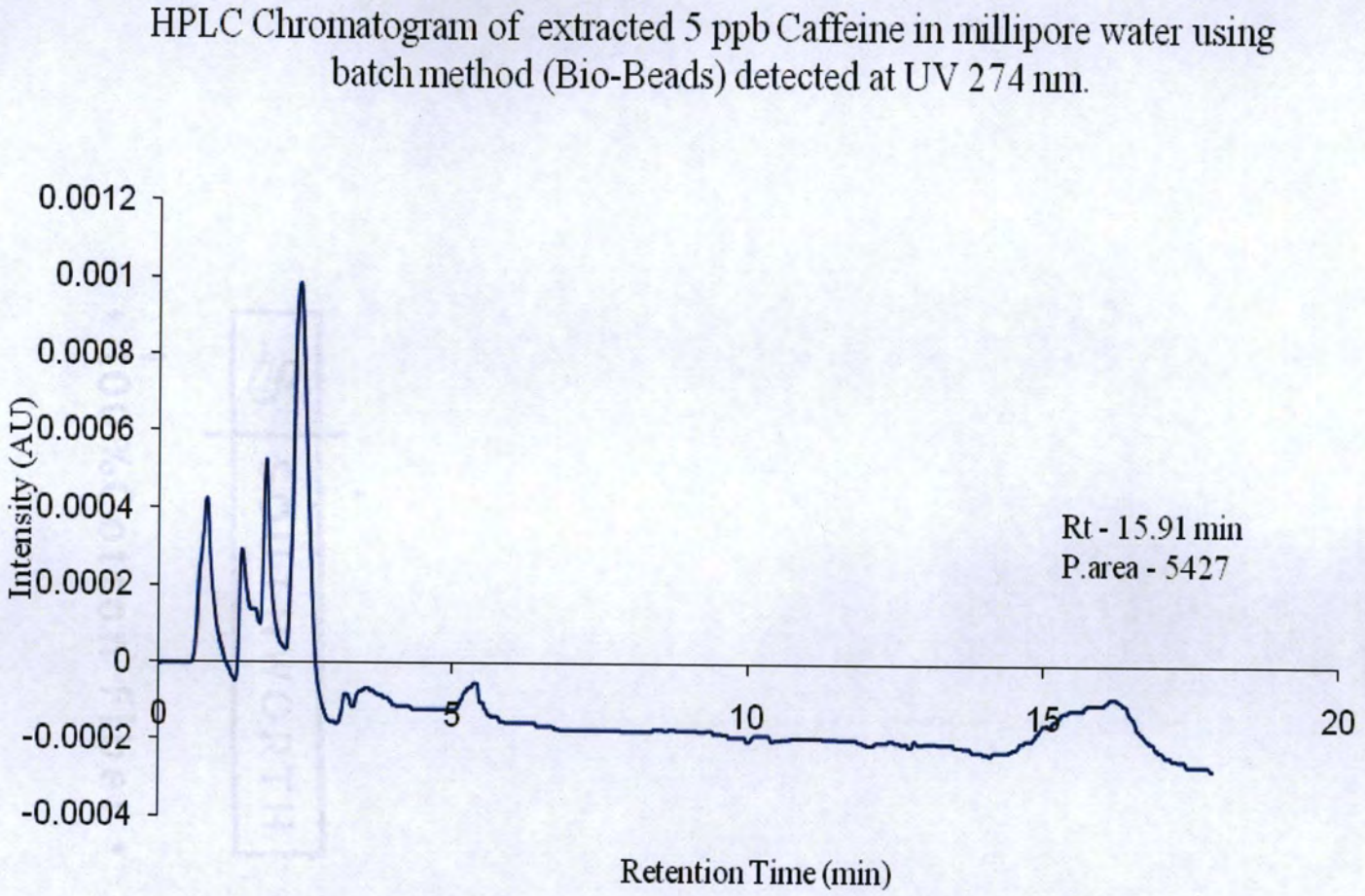


Figure 10 (d): HPLC chromatogram of extracted 5 ppb caffeine in millipore water using Bio-Beads batch method (detected at UV wavelength of 274 nm).

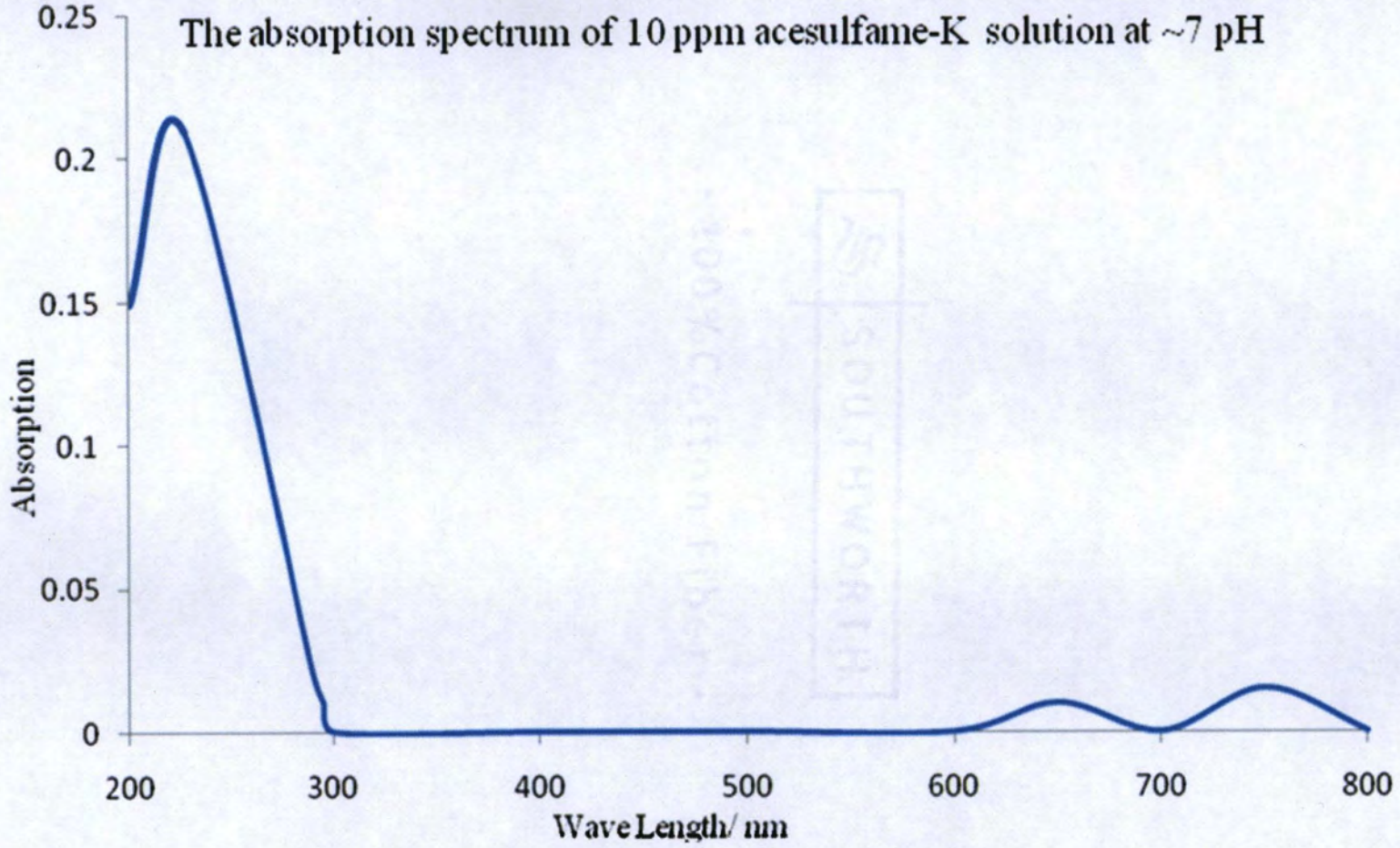


Figure 11: The UV absorption spectrum of 10 ppm acesulfame-K solution at ~7 pH

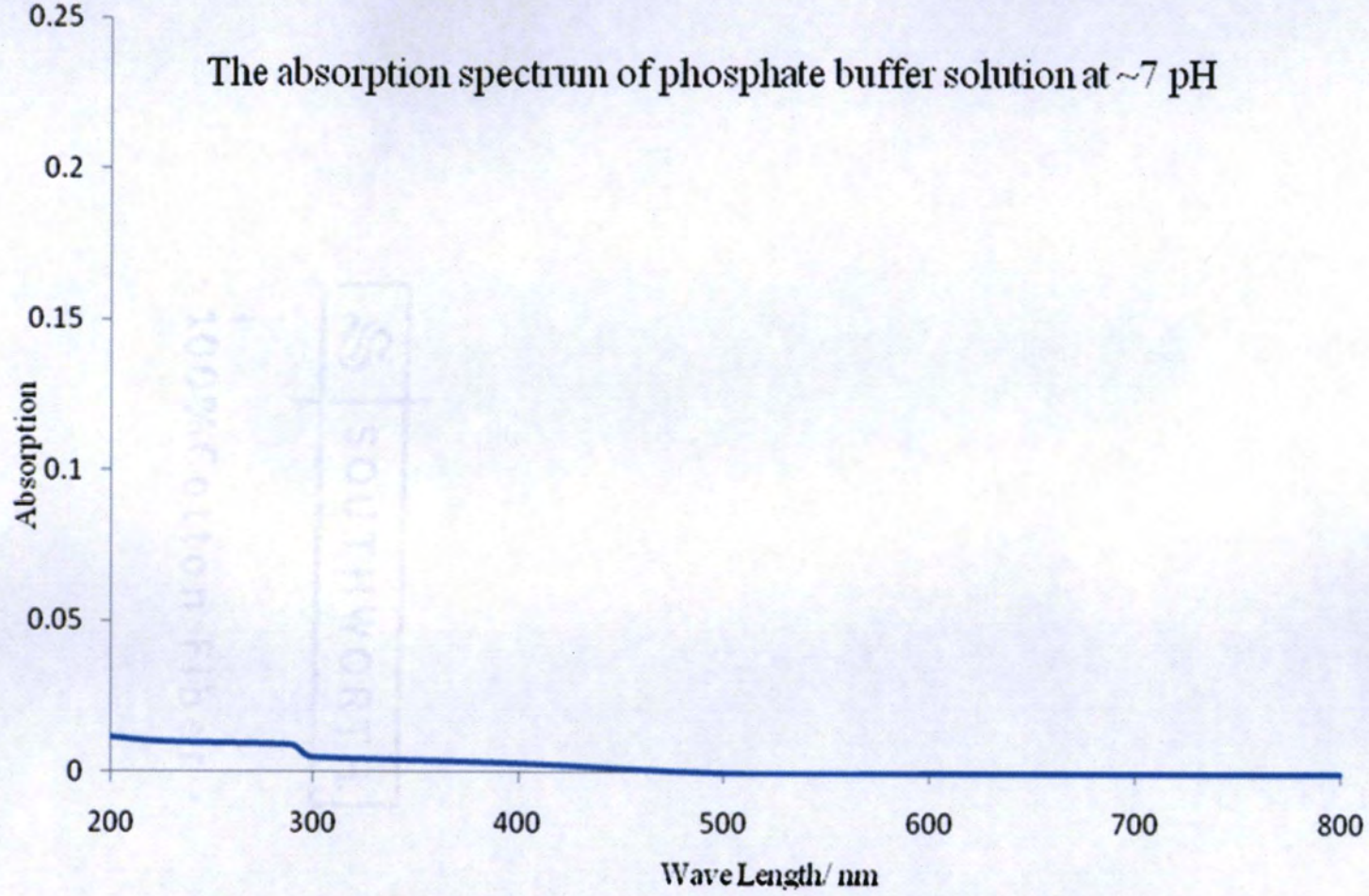


Figure 12: The UV absorption spectrum of phosphate buffer solution at ~7 pH

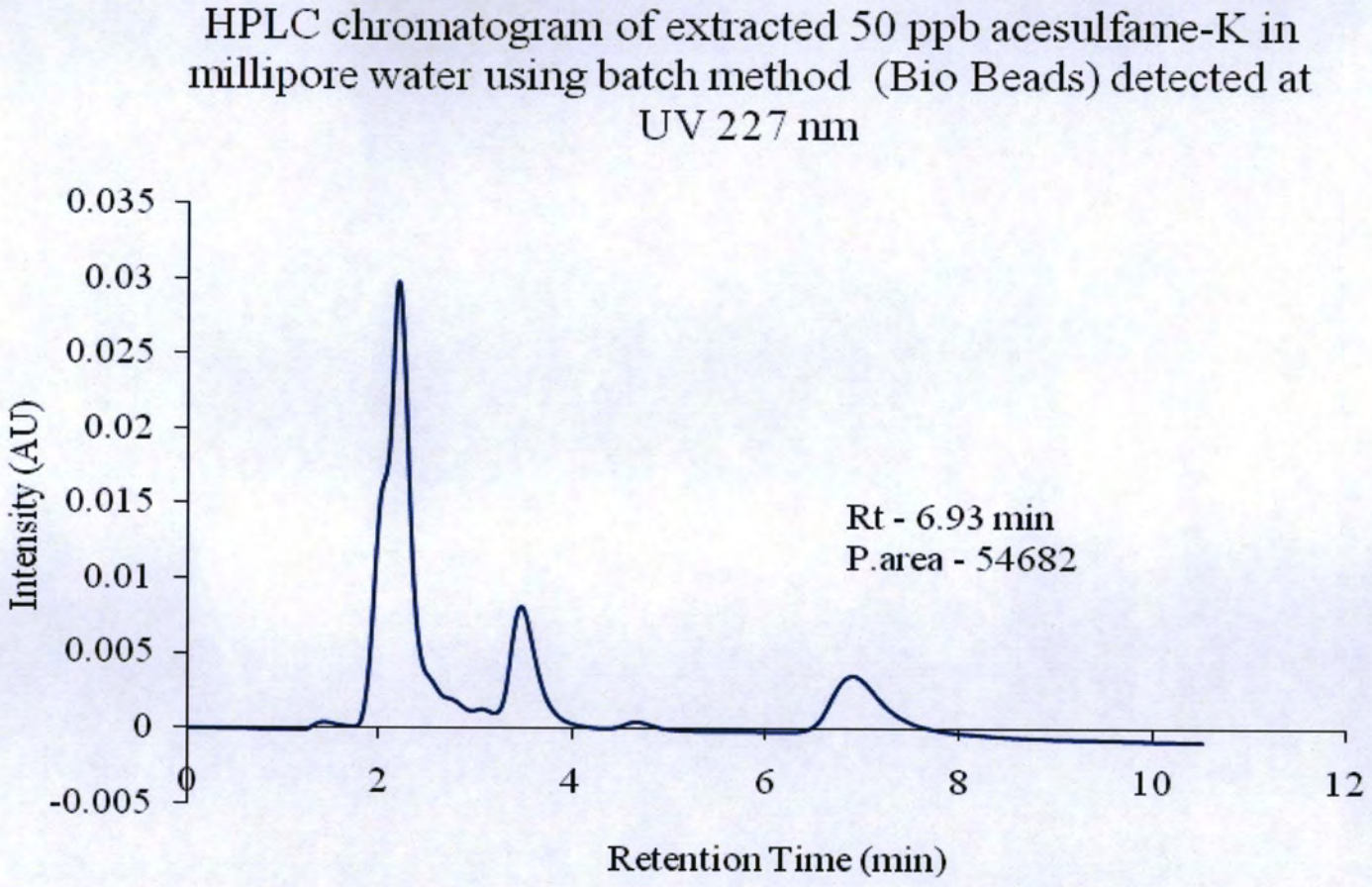


Figure 13 (a): HPLC chromatogram of extracted 50 ppb acesulfame-K in millipore water using Bio-Beads batch method (detected at UV wavelength of 227 nm).

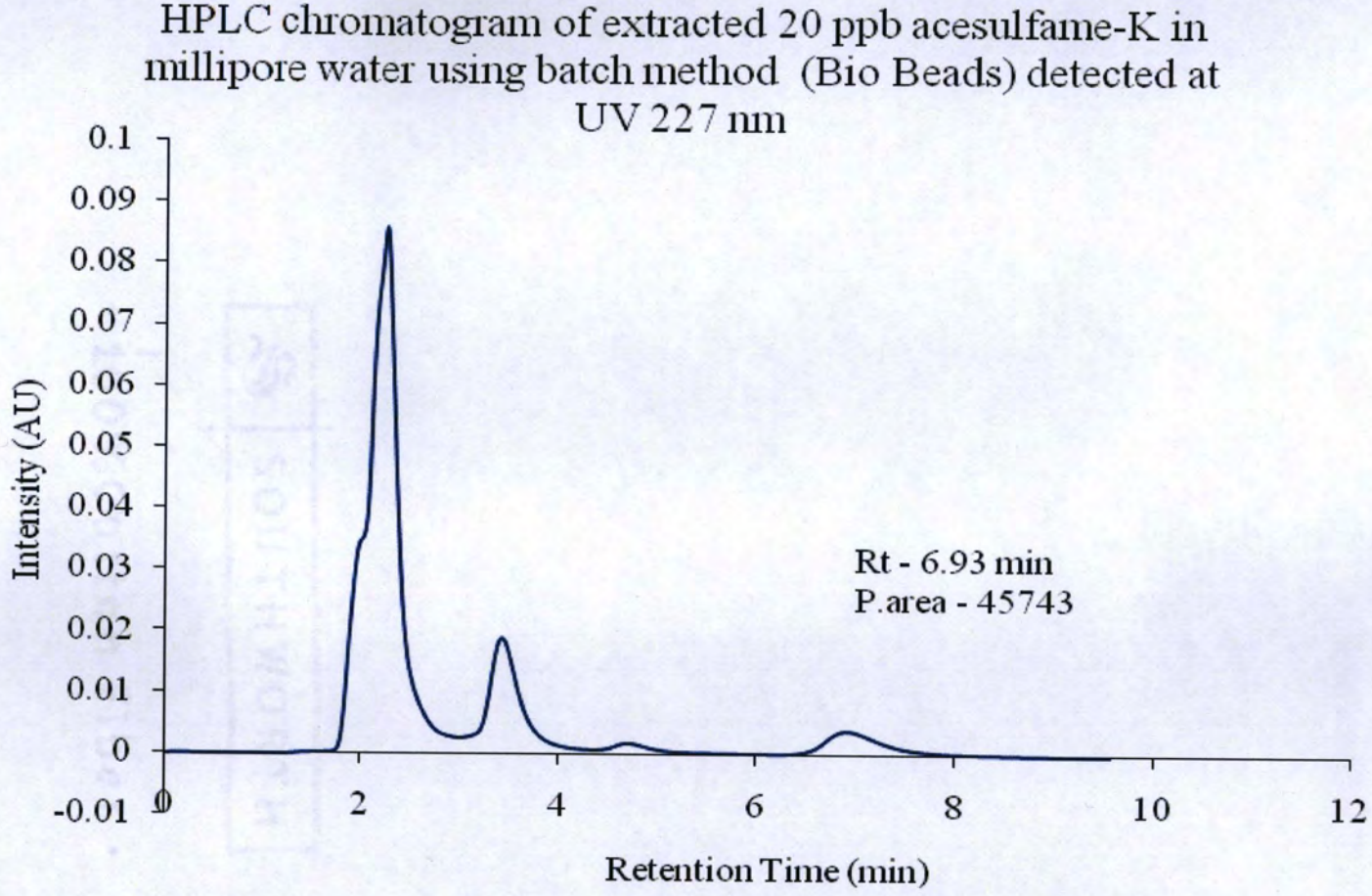


Figure 13 (b): HPLC chromatogram of extracted 20 ppb acesulfame-K in millipore water using Bio-Beads batch method (detected at UV wavelength of 227 nm).

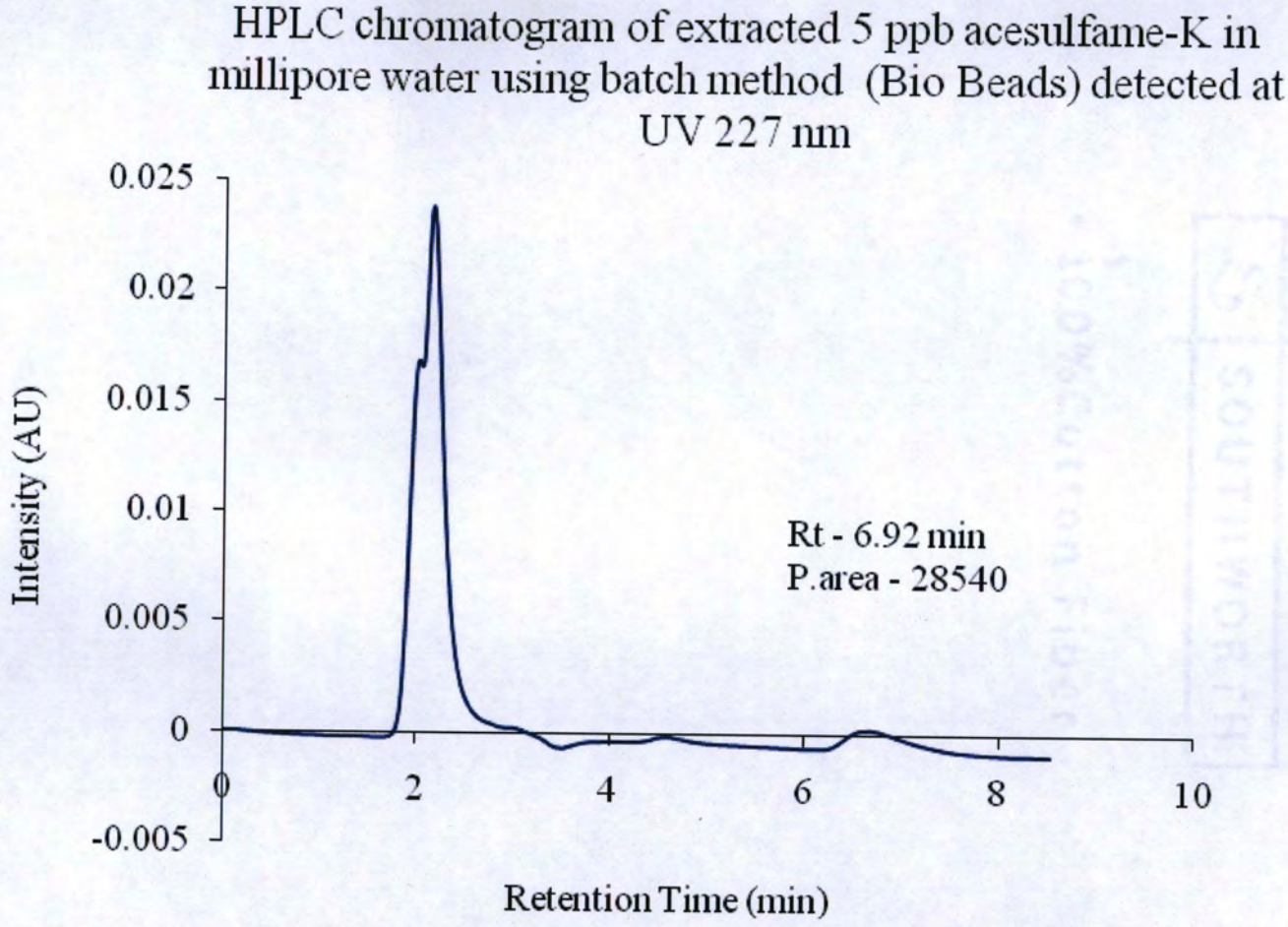


Figure 13 (c): HPLC chromatogram of extracted 5 ppb acesulfame-K in millipore water using Bio-Beads batch method (detected at UV wavelength of 227 nm).

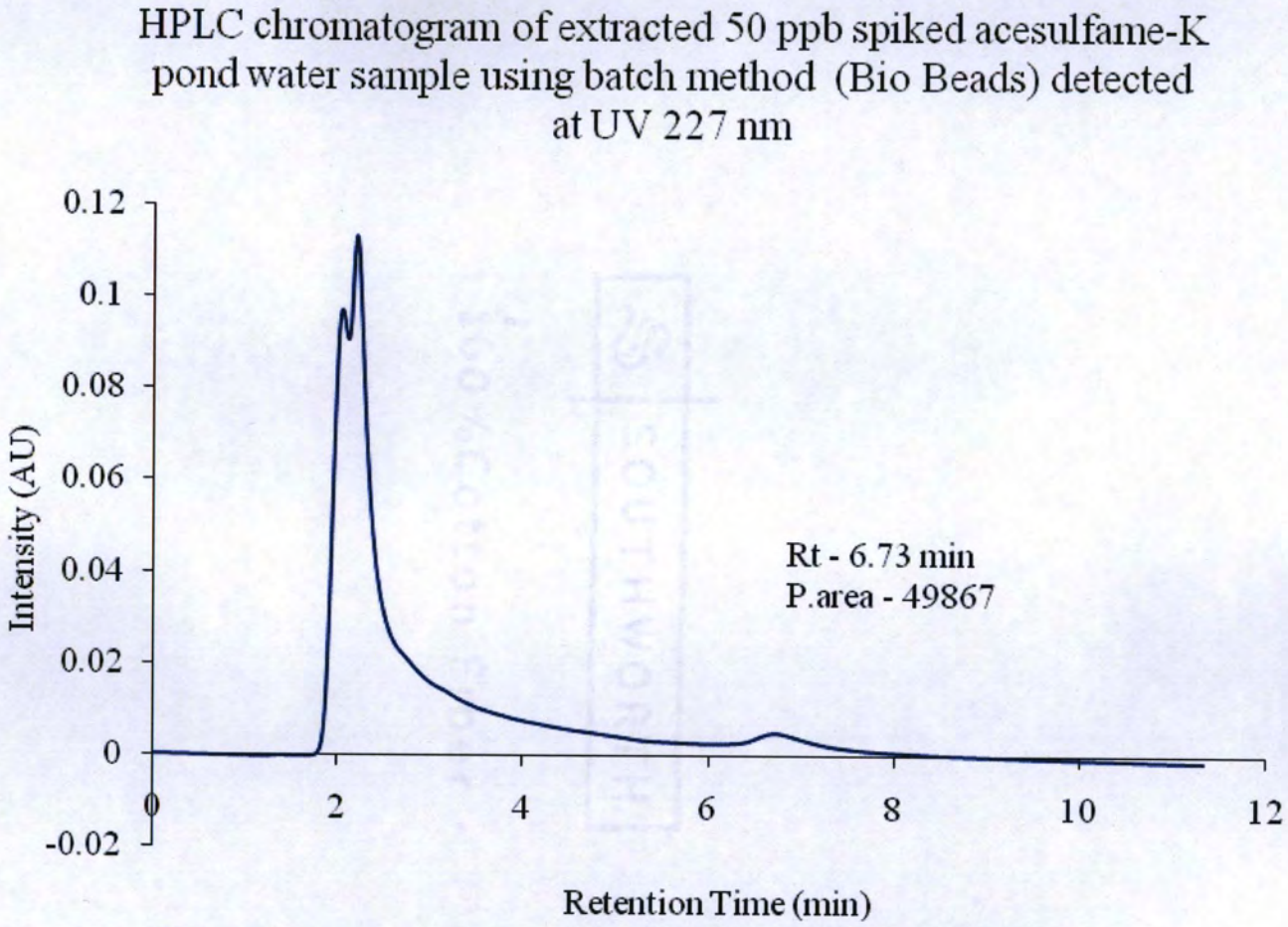


Figure 14: HPLC chromatogram of extracted 50 ppb acesulfame-K spiked pond water sample using Bio-Beads batch method (detected at UV wavelength of 227 nm).

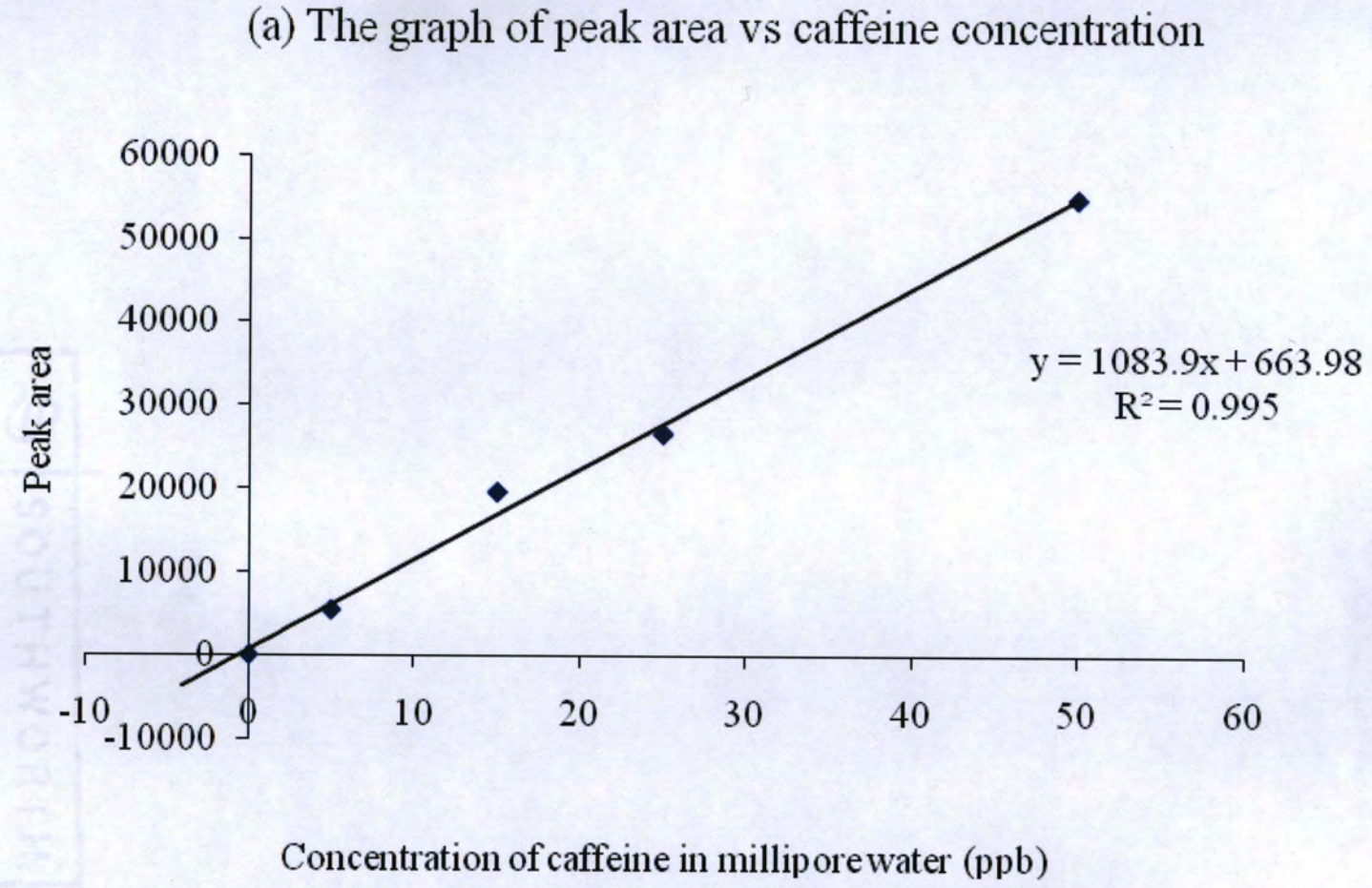


Figure 15 (a): The graph of peak area vs concentration of caffeine in millipore water

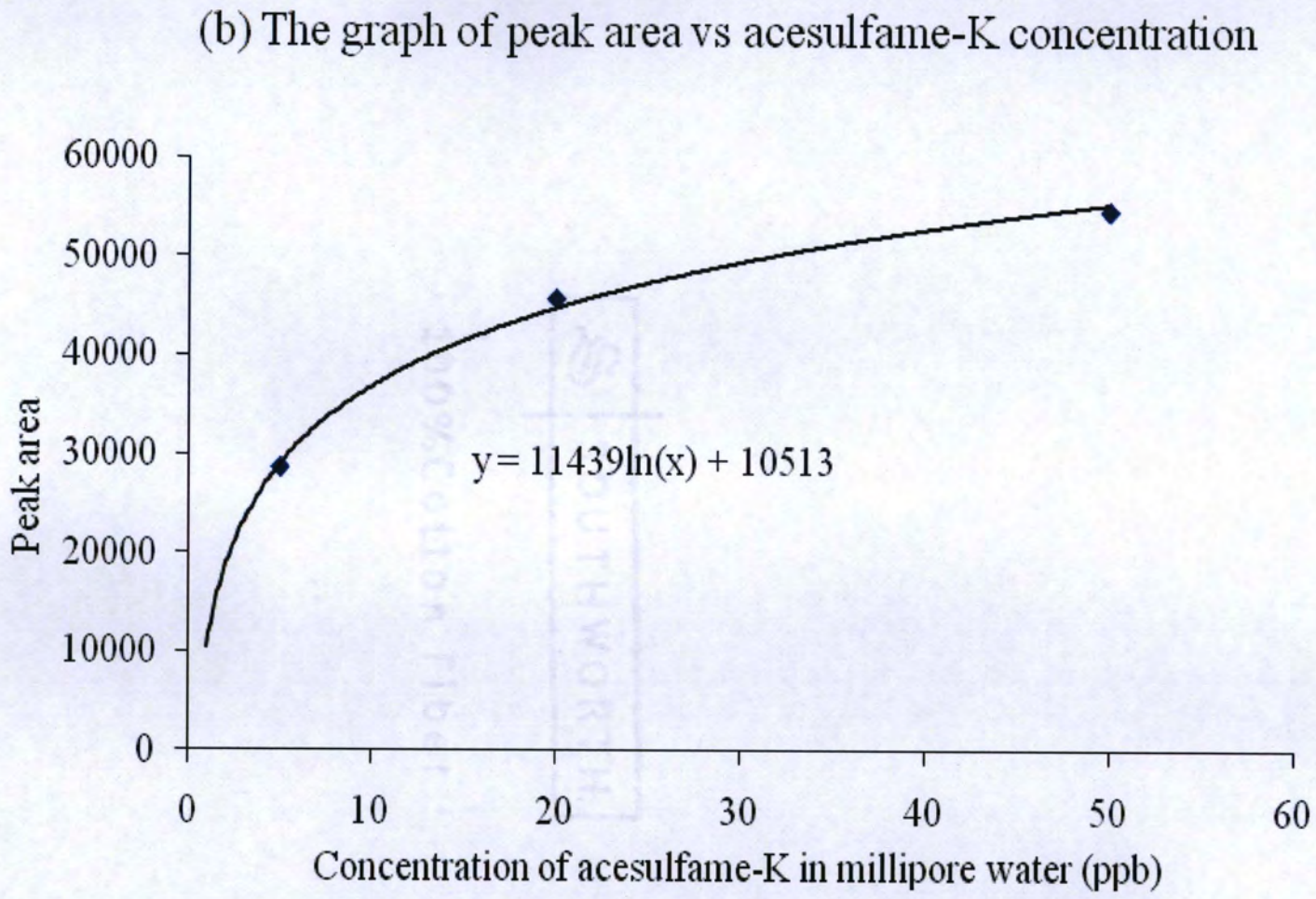


Figure 15 (b): The graph of peak area vs concentration of acesulfame-K in millipore water

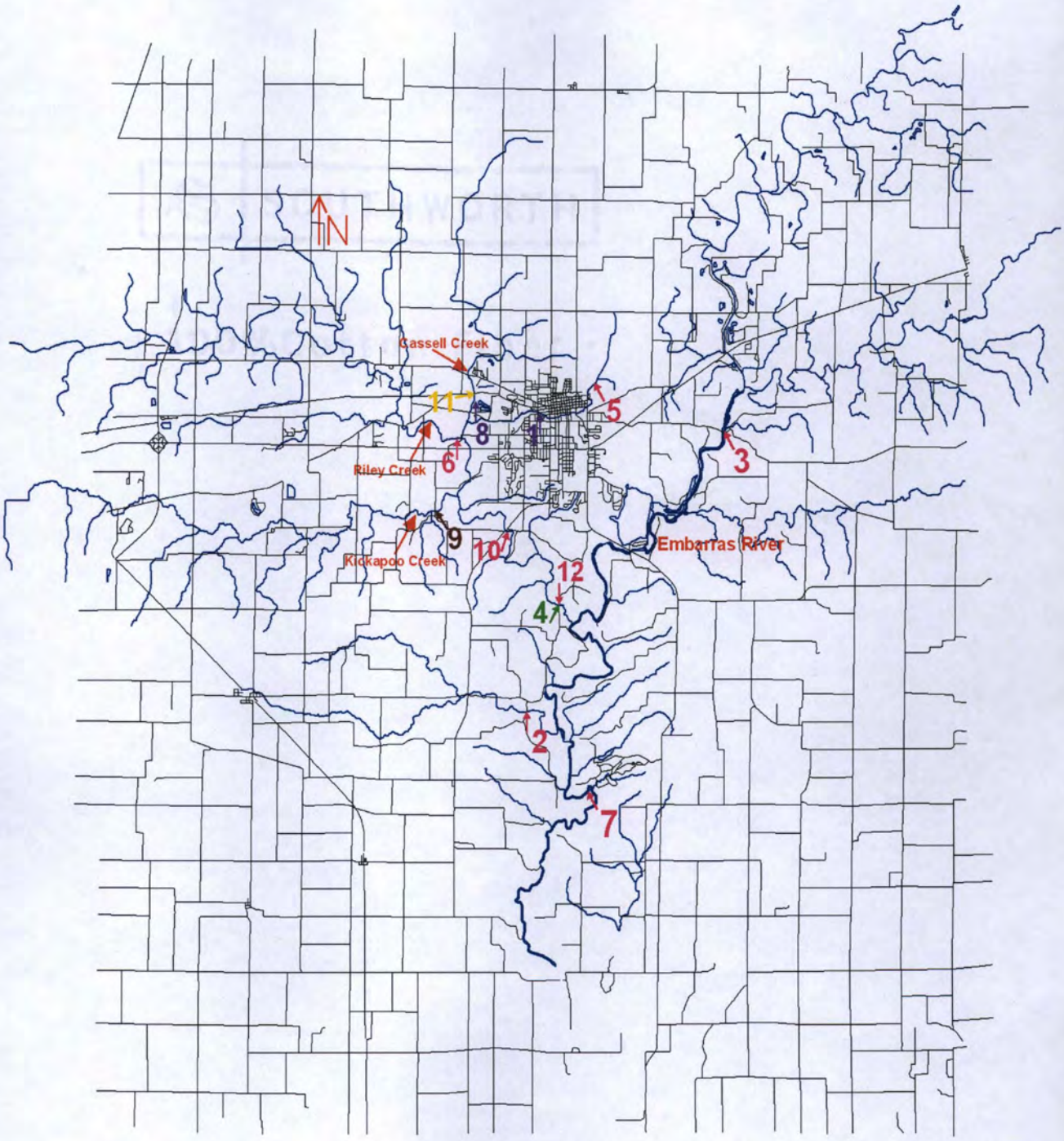


Figure 16: Map of sampling sites in Charleston City area. (Purple = area of only acesulfame-K detected, Pink = area of detected non, Yellow = area of only caffeine detected, Green = area of detected both)

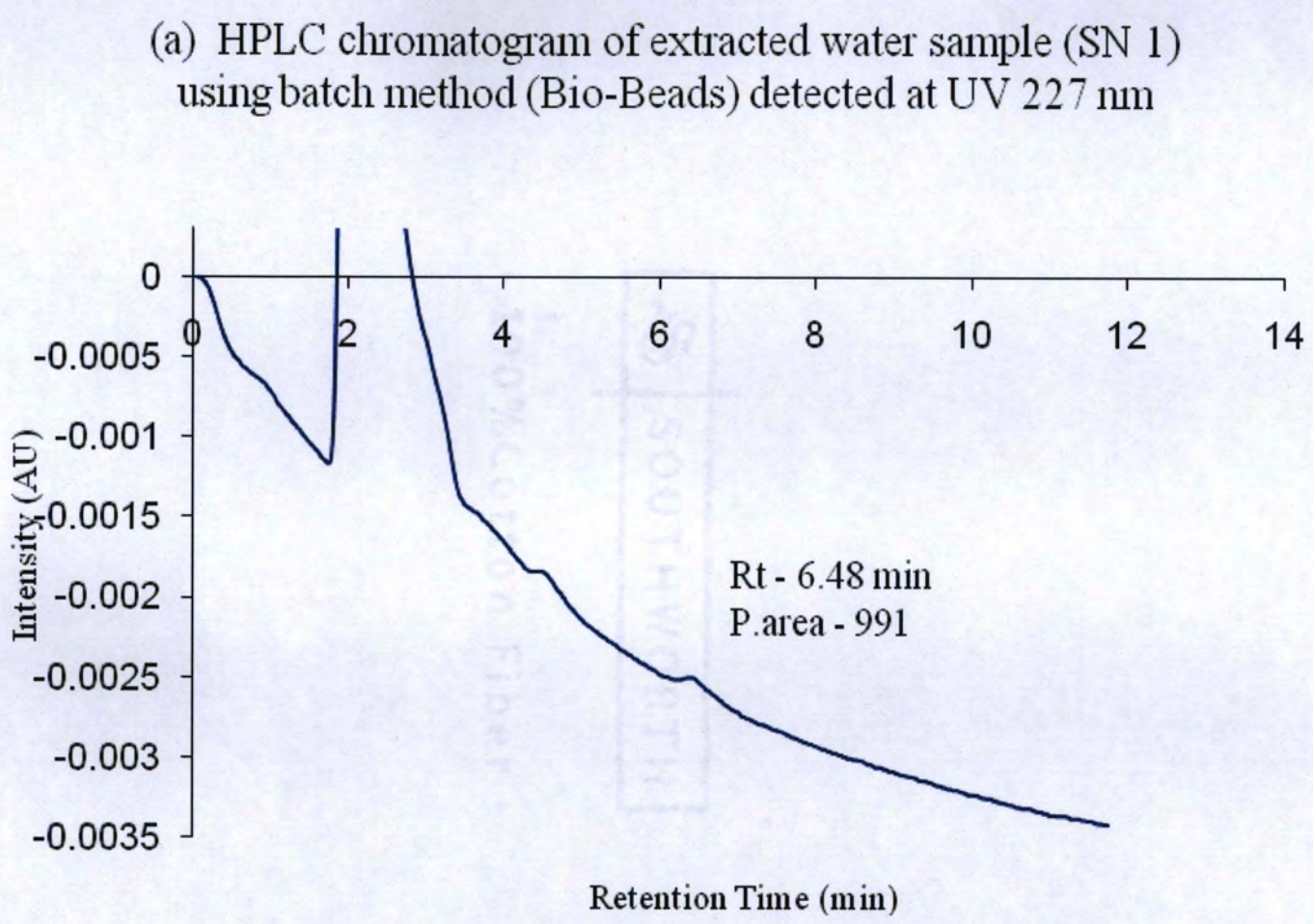


Figure 17 (a): HPLC chromatogram of extracted water sample no 1 using Bio-Beads batch method (detected at UV wavelength of 227 nm – for acesulfame-K).

(b) HPLC chromatogram of extracted water sample (SN 4) using batch method (Bio-Beads) detected at UV 227 nm

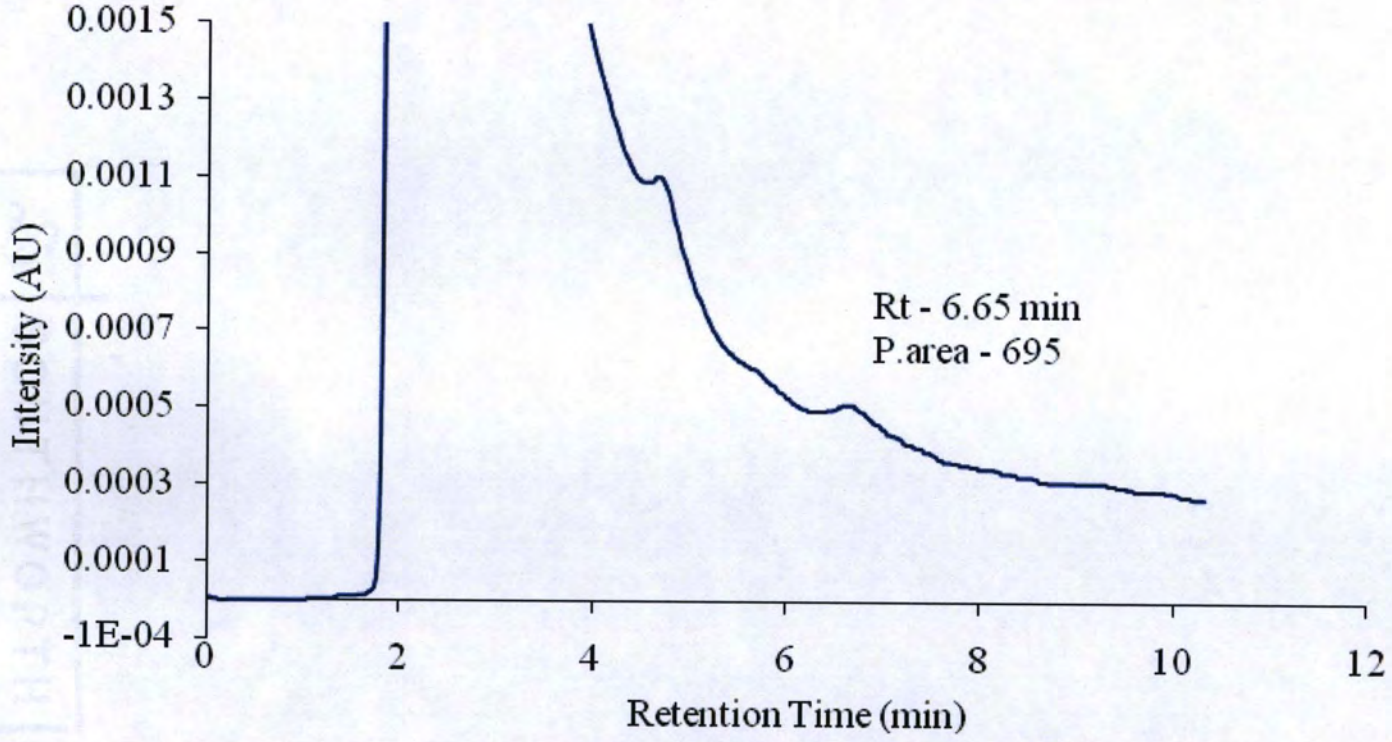


Figure 17 (b): HPLC chromatogram of extracted water sample no 4 using Bio-Beads batch method (detected at UV wavelength of 227 nm – for acesulfame-K).

(c) HPLC chromatogram of extracted water sample (SN 8) using batch method (Bio-Beads) detected at UV 227 nm

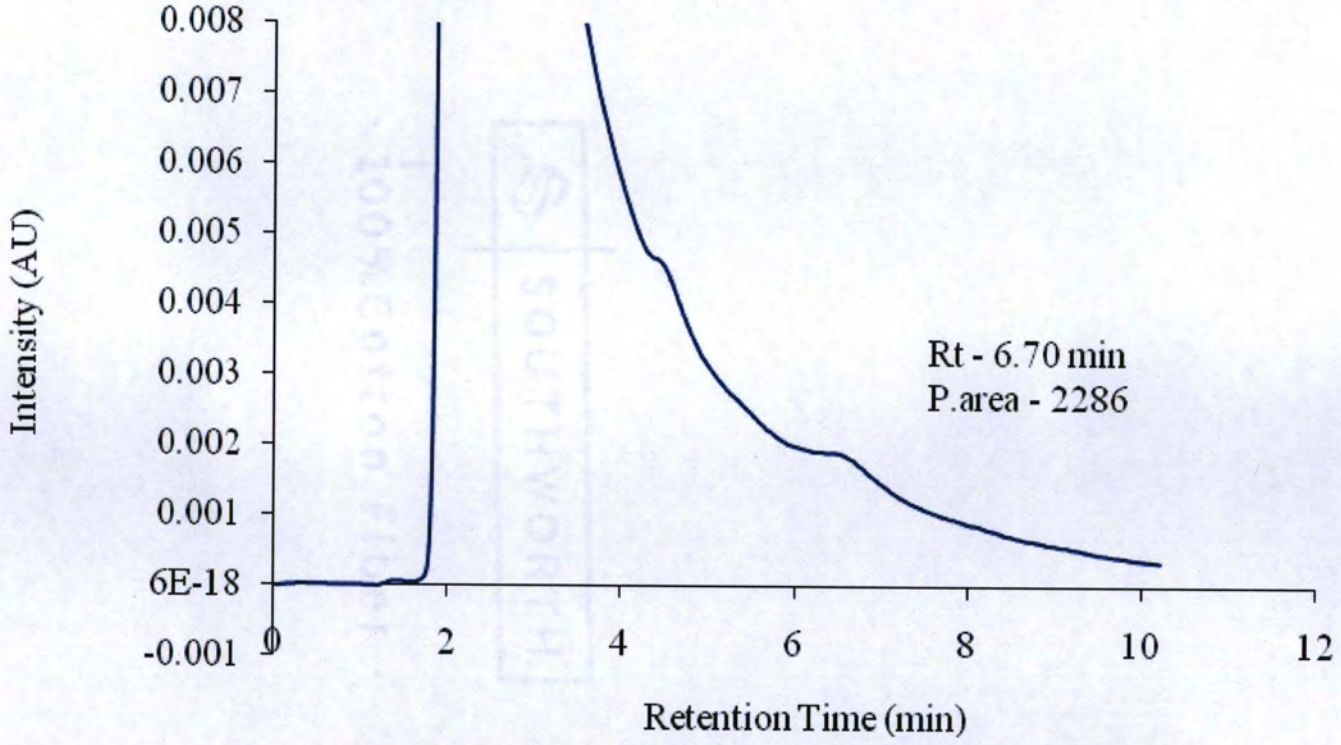


Figure 17 (c): HPLC chromatogram of extracted water sample no 8 using Bio-Beads batch method (detected at UV wavelength of 227 nm – for acesulfame-K).

(a) HPLC chromatogram of extracted 5 ppb acesulfame-K in millipore water using batch method (Bio Beads) detected at UV 227 nm

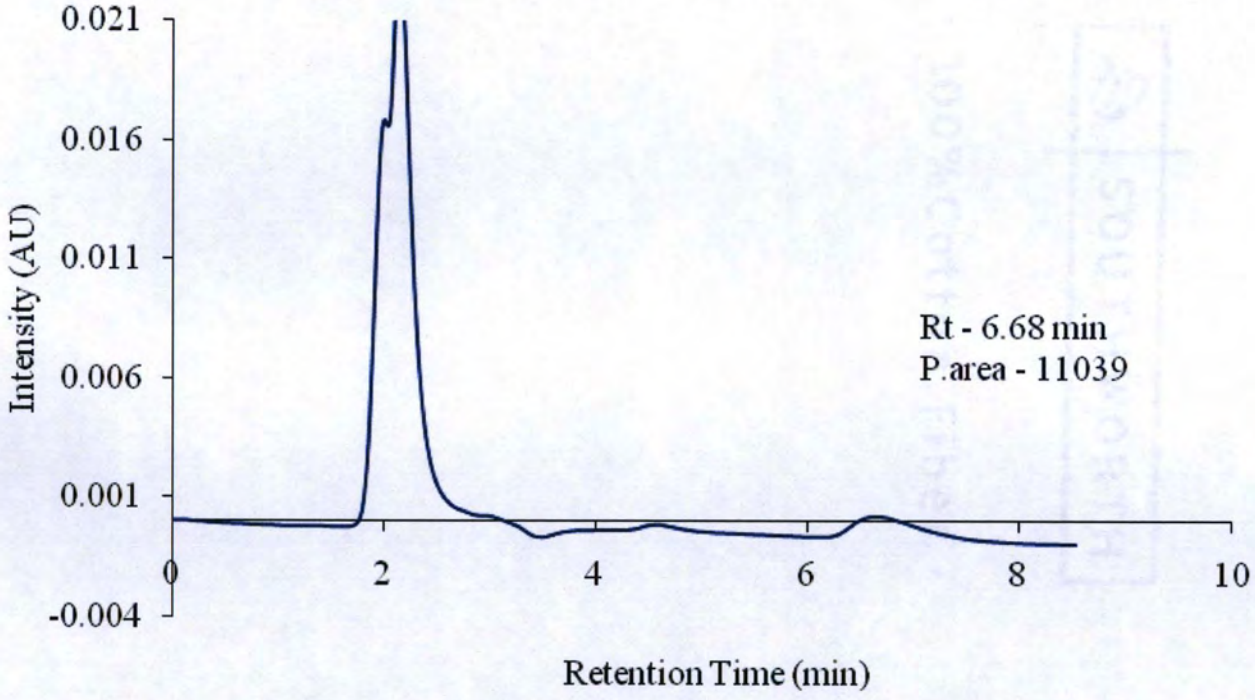


Figure 18 (a): HPLC chromatogram of extracted 5 ppb acesulfame-K in millipore water using Bio-Beads batch method (detected at UV wavelength of 227 nm) to compare with the chromatogram of SN 1.

(b) HPLC chromatogram of extracted 5 ppb acesulfame-K in millipore water using batch method (Bio Beads) detected at UV 227 nm

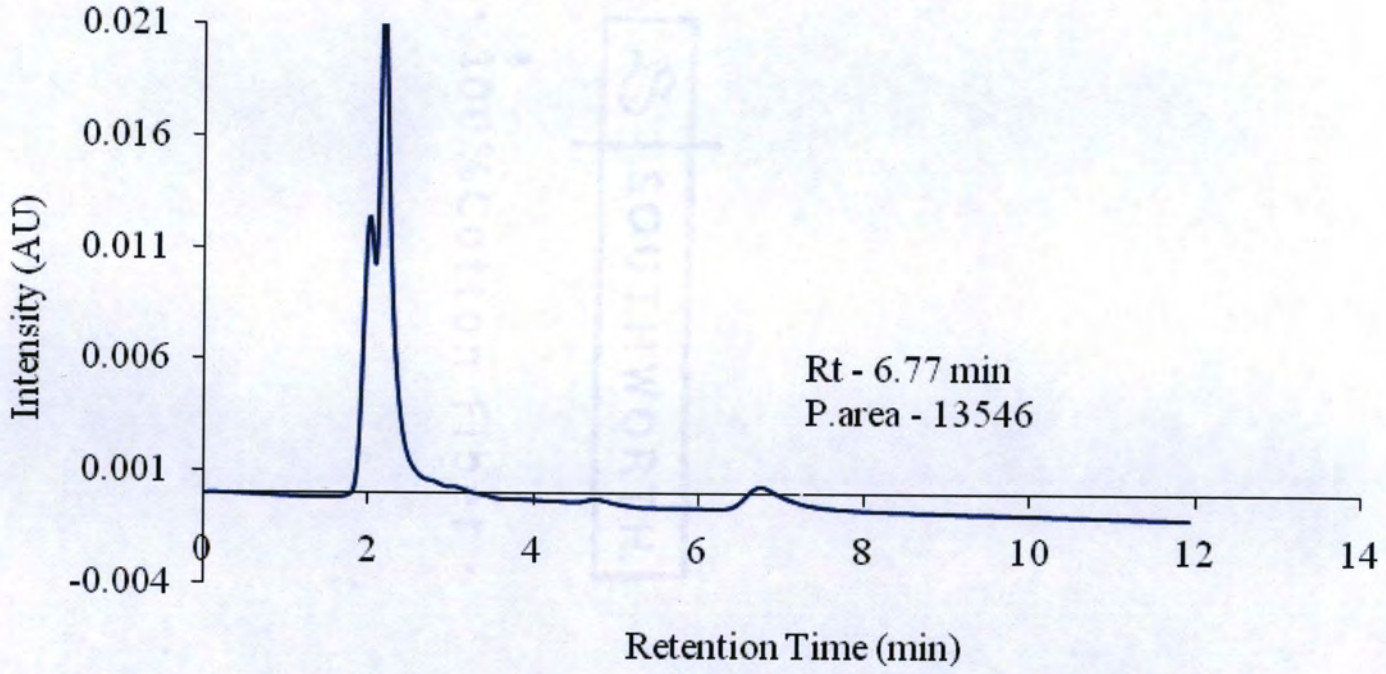


Figure 18 (b): HPLC chromatogram of extracted 5 ppb acesulfame-K in millipore water using Bio-Beads batch method (detected at UV wavelength of 227 nm) to compare with the chromatogram of SN 4.

(c) HPLC chromatogram of extracted 5 ppb acesulfame-K in millipore water using batch method (Bio Beads) detected at UV 227 nm

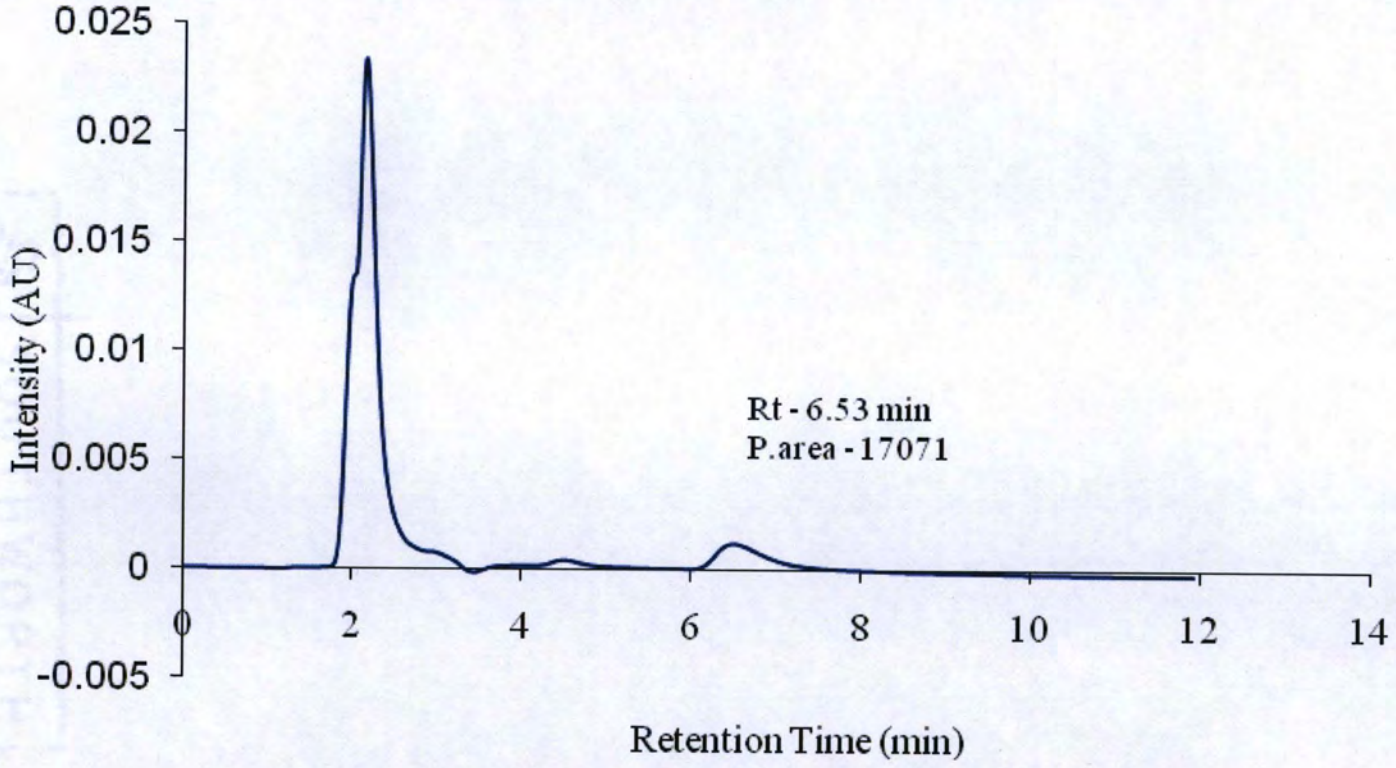


Figure 18 (c): HPLC chromatogram of extracted 5 ppb acesulfame-K in millipore water using Bio-Beads batch method (detected at UV wavelength of 227 nm) to compare with the chromatogram of SN 8.

(a) HPLC chromatogram of extracted water sample (SN 4) using batch method (Bio-Beads) detected at UV 274 nm

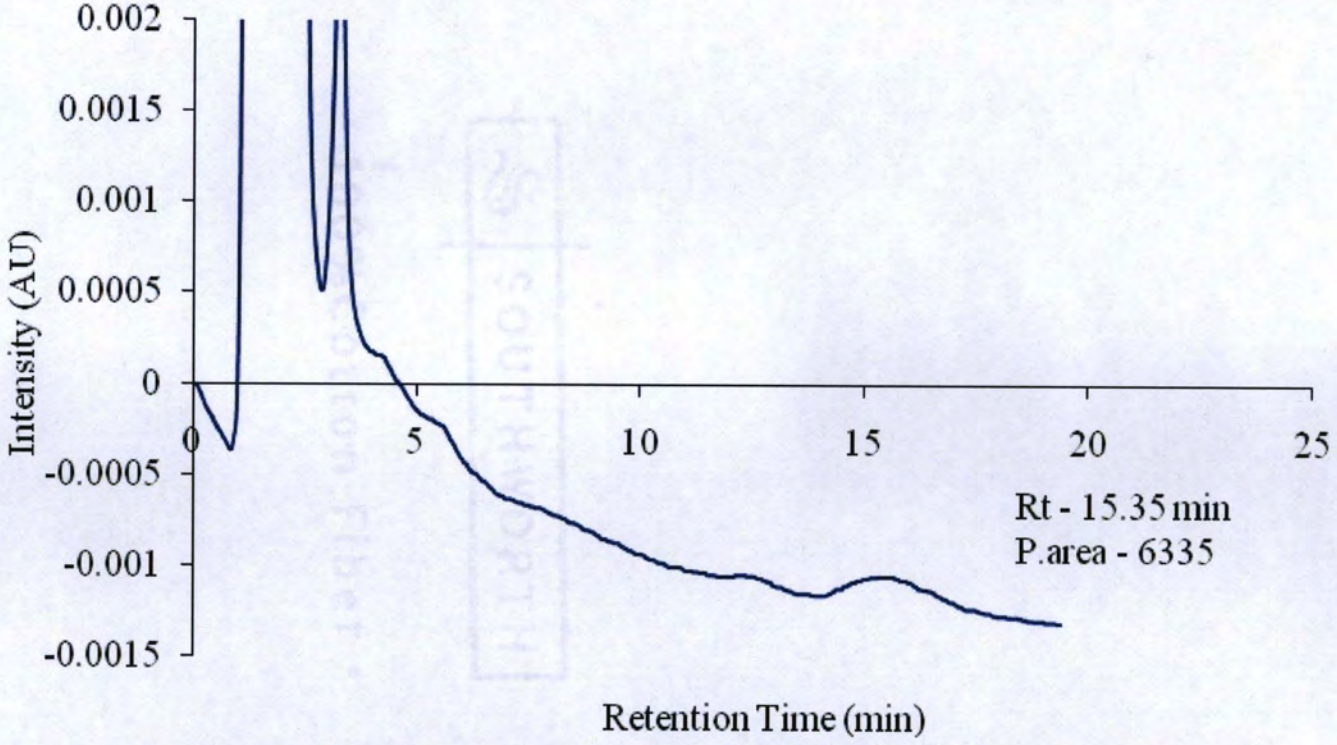


Figure 19 (a): HPLC chromatogram of extracted water sample no 4 using Bio-Beads batch method (detected at UV wavelength of 274 nm – for caffeine).

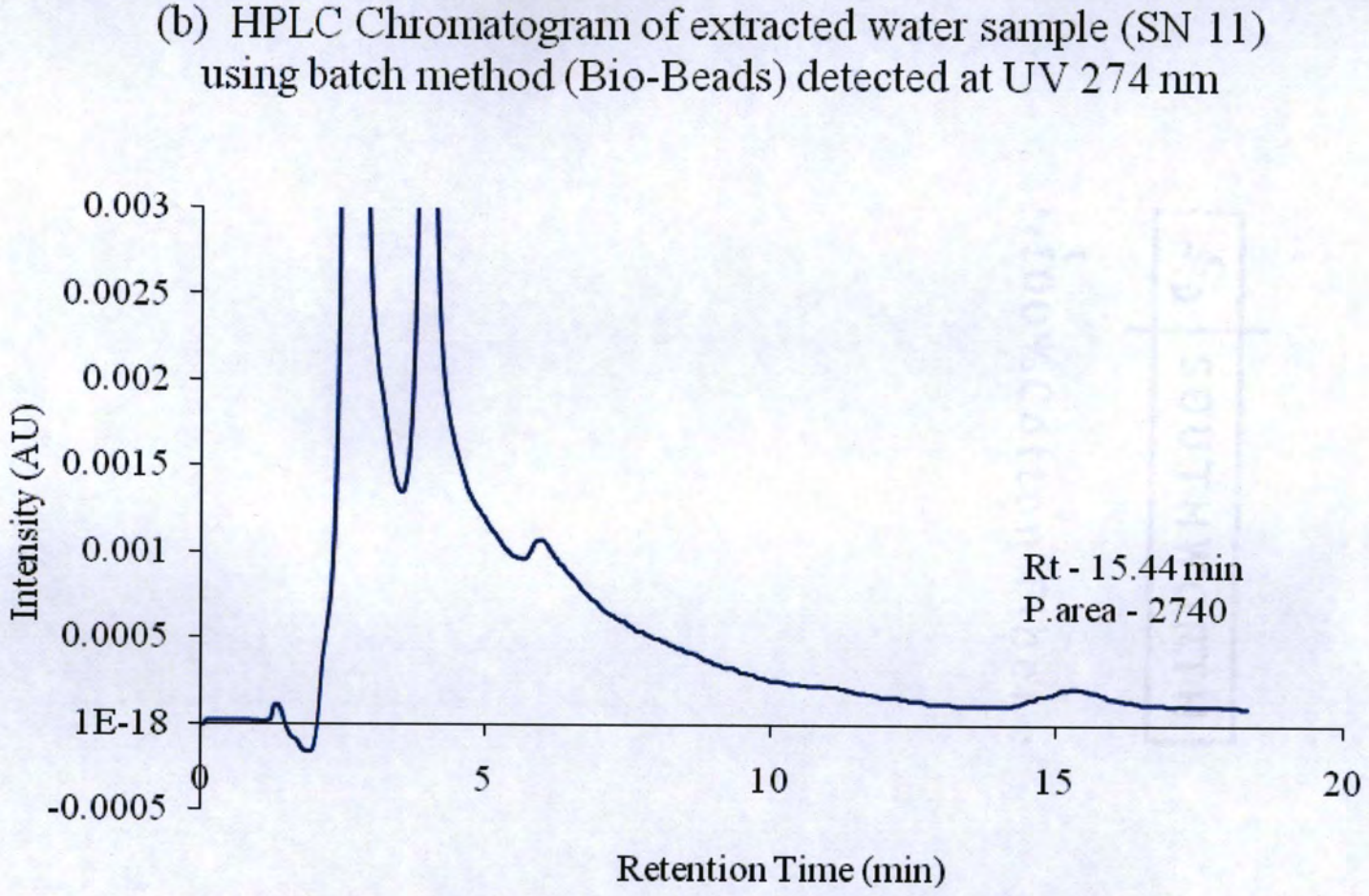


Figure 19 (b): HPLC chromatogram of extracted water sample no 11 using Bio-Beads batch method (detected at UV wavelength of 274 nm – for caffeine).

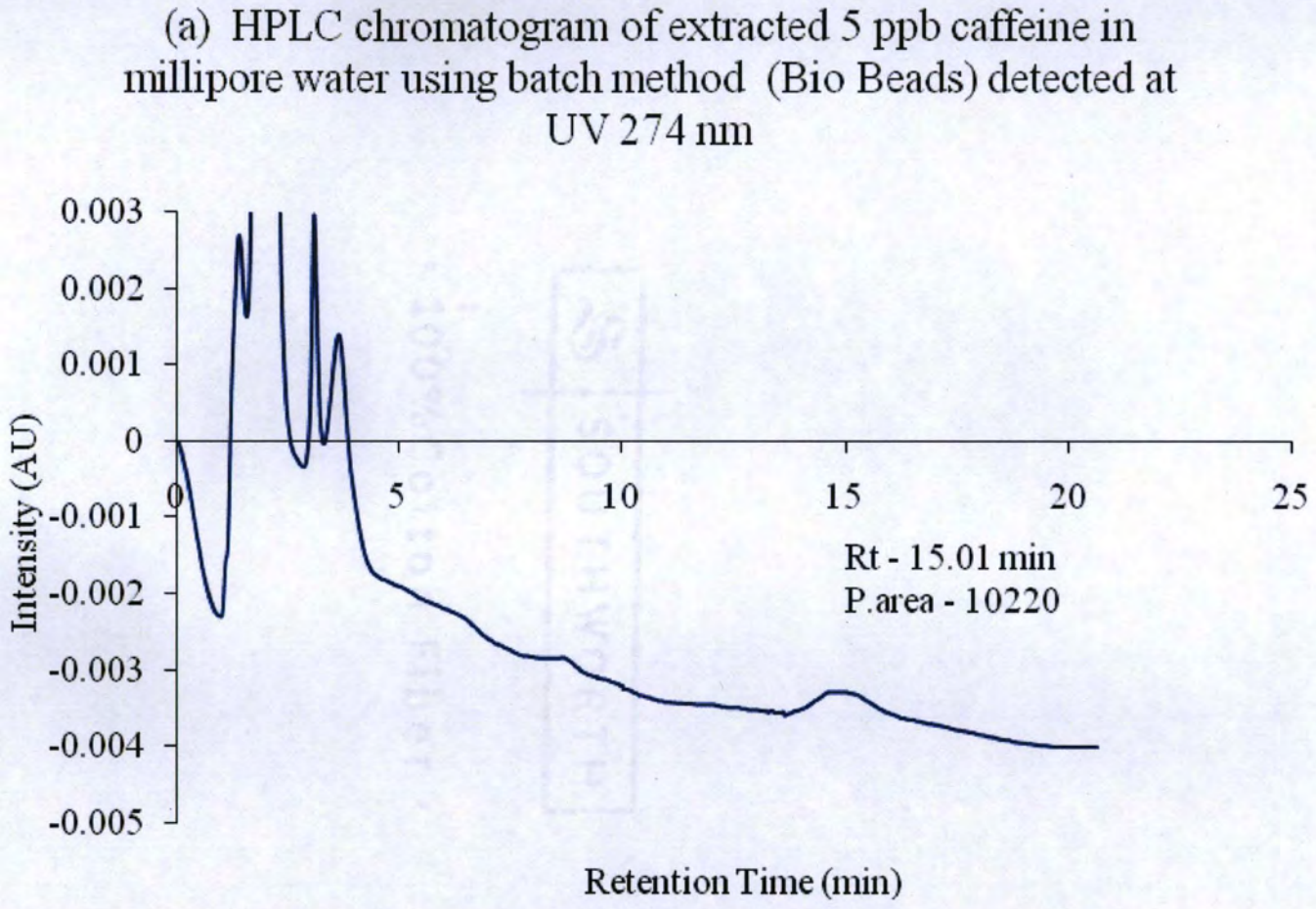


Figure 20 (a): HPLC chromatogram of extracted 5 ppb caffeine in millipore water using Bio-Beads batch method (detected at UV wavelength of 274 nm) to compare with the chromatogram of SN 4.

(b) HPLC chromatogram of extracted 5 ppb caffeine in millipore water using batch method (Bio Beads) detected at UV 274 nm

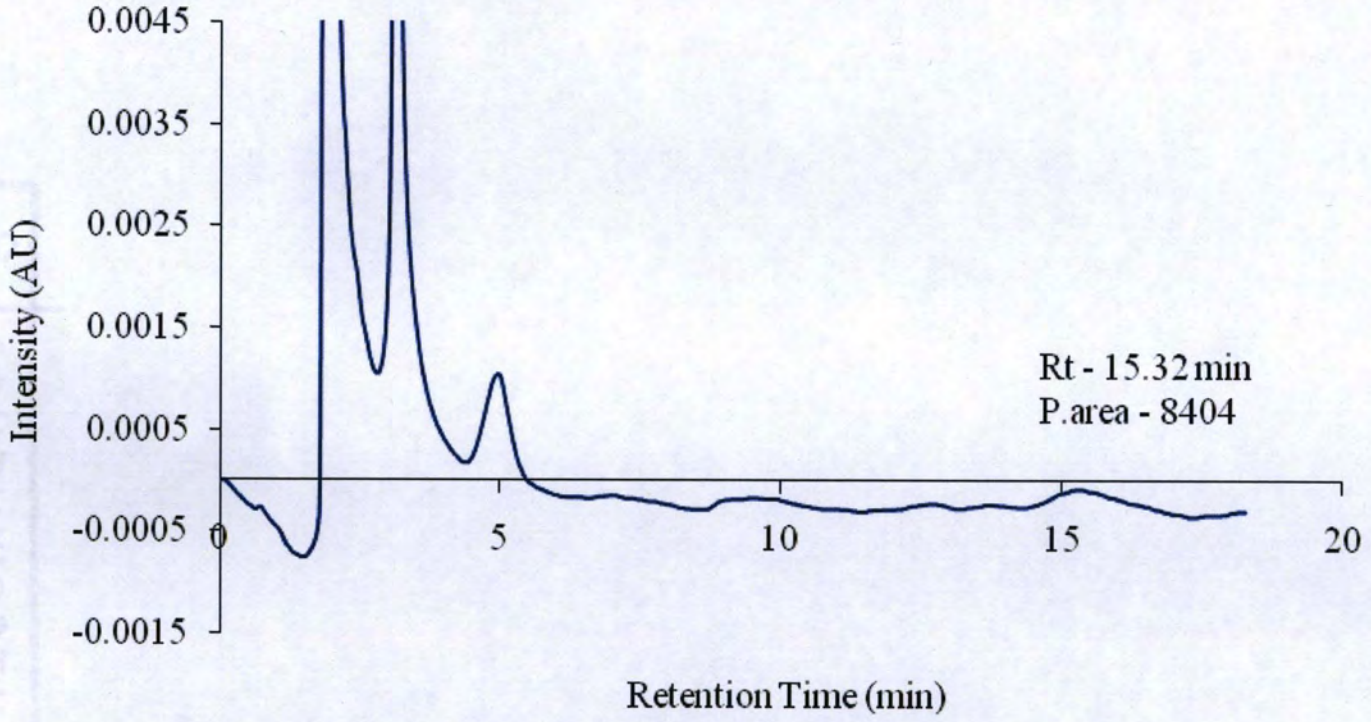
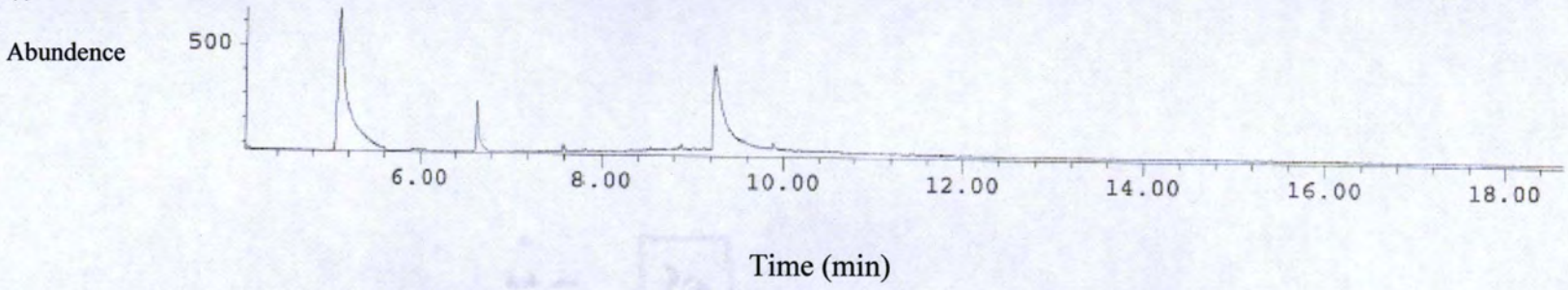


Figure 20 (b): HPLC chromatogram of extracted 5 ppb caffeine in millipore water using Bio-Beads batch method (detected at UV wavelength of 274 nm) to compare with the chromatogram of SN 11.

(i)



(ii)

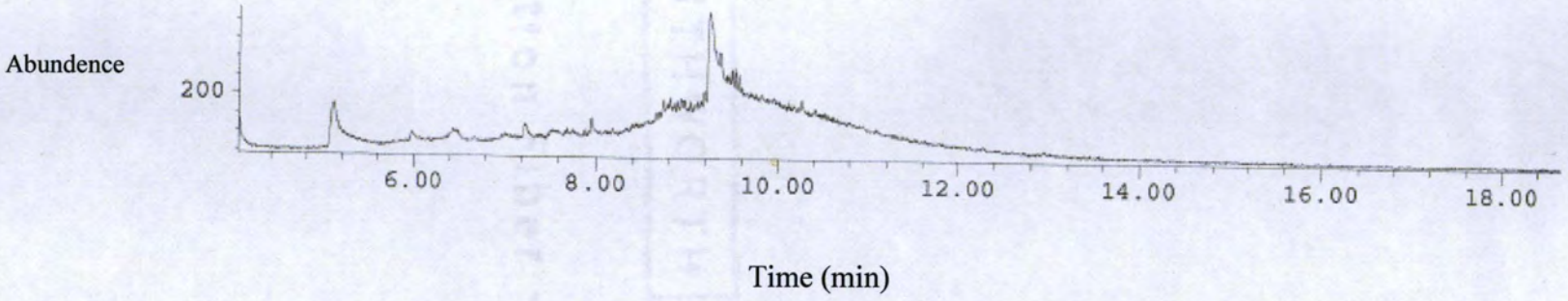
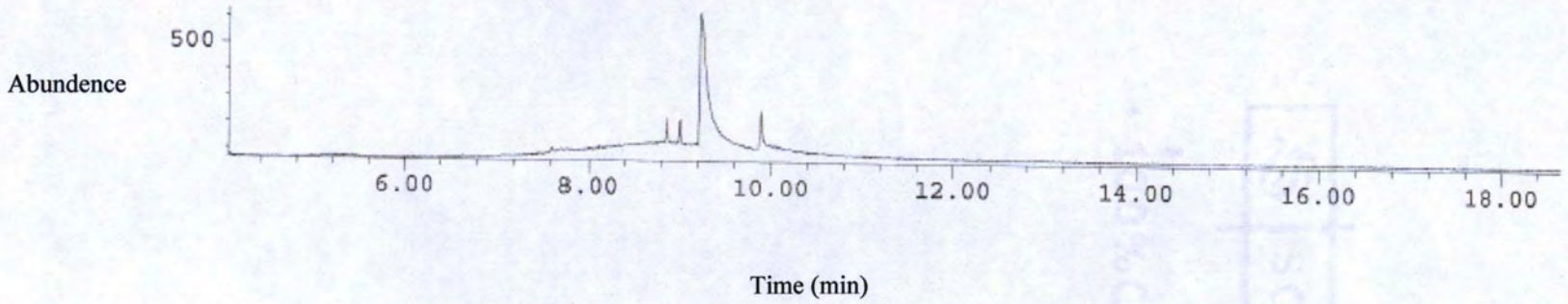


Figure 21 (a): SIM GC/MS chromatogram of extracted water sample no 11 using Bio-Beads batch method. (i) The chromatogram of 194.1 SIM and (ii) The chromatogram of 109 SIM (The peak at 9.3 min shows the presence of caffeine in both graphs)

(i)



(ii)

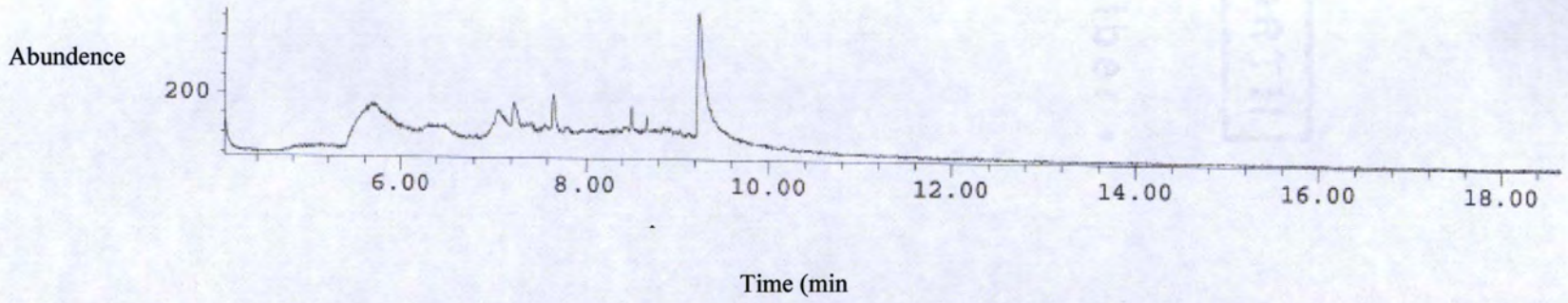


Figure 21 (b): SIM GC/MS chromatogram of extracted 50 ppb caffeine in millipore water using Bio-Beads batch method. (i) The chromatogram of 194.1 SIM and (ii) The chromatogram of 109 SIM (The peak at 9.3 min shows the presence of caffeine in both graphs)

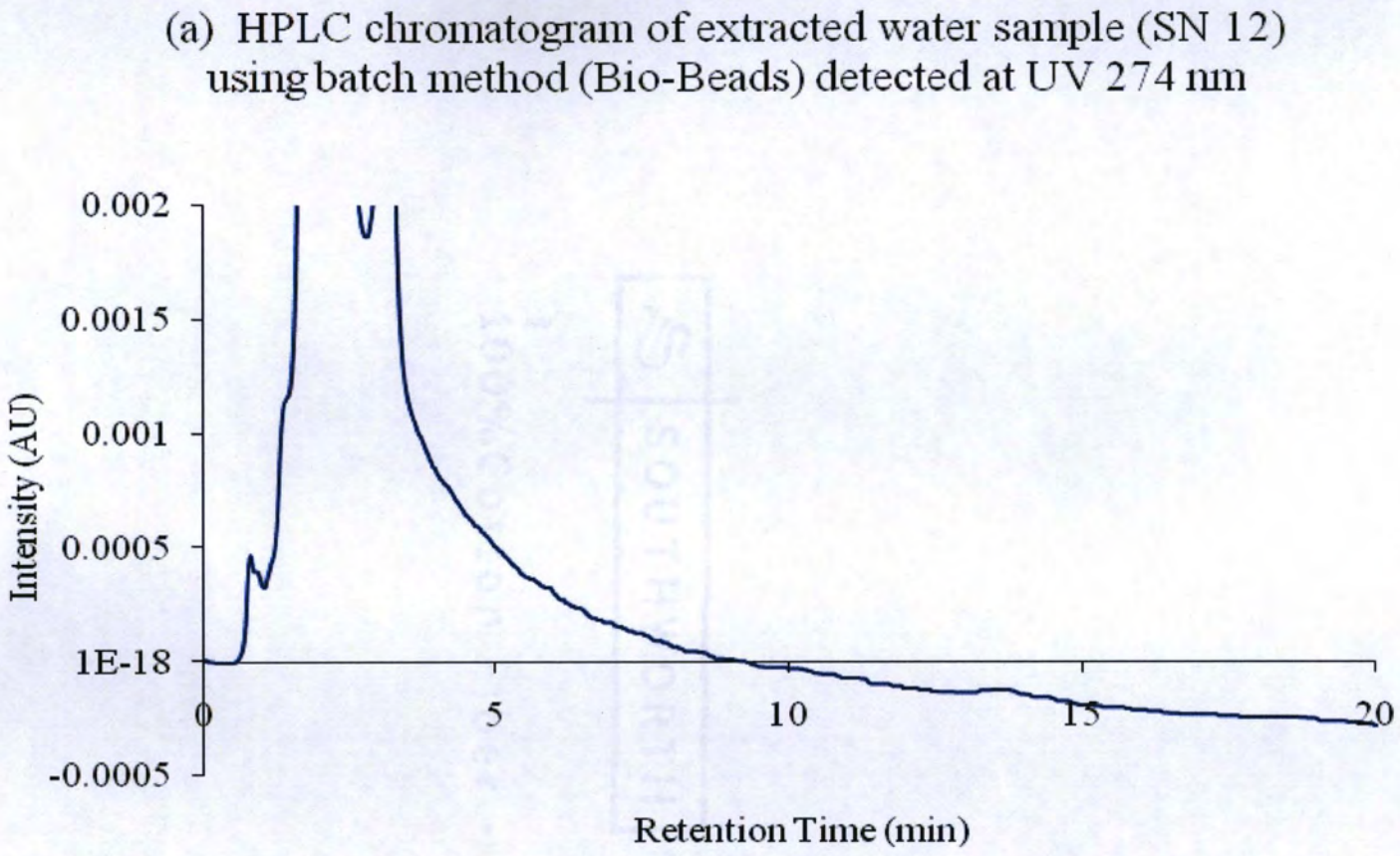


Figure 22 (a): HPLC chromatogram of extracted water sample no 12 using Bio-Beads batch method (detected at UV wavelength of 274 nm – for caffeine).

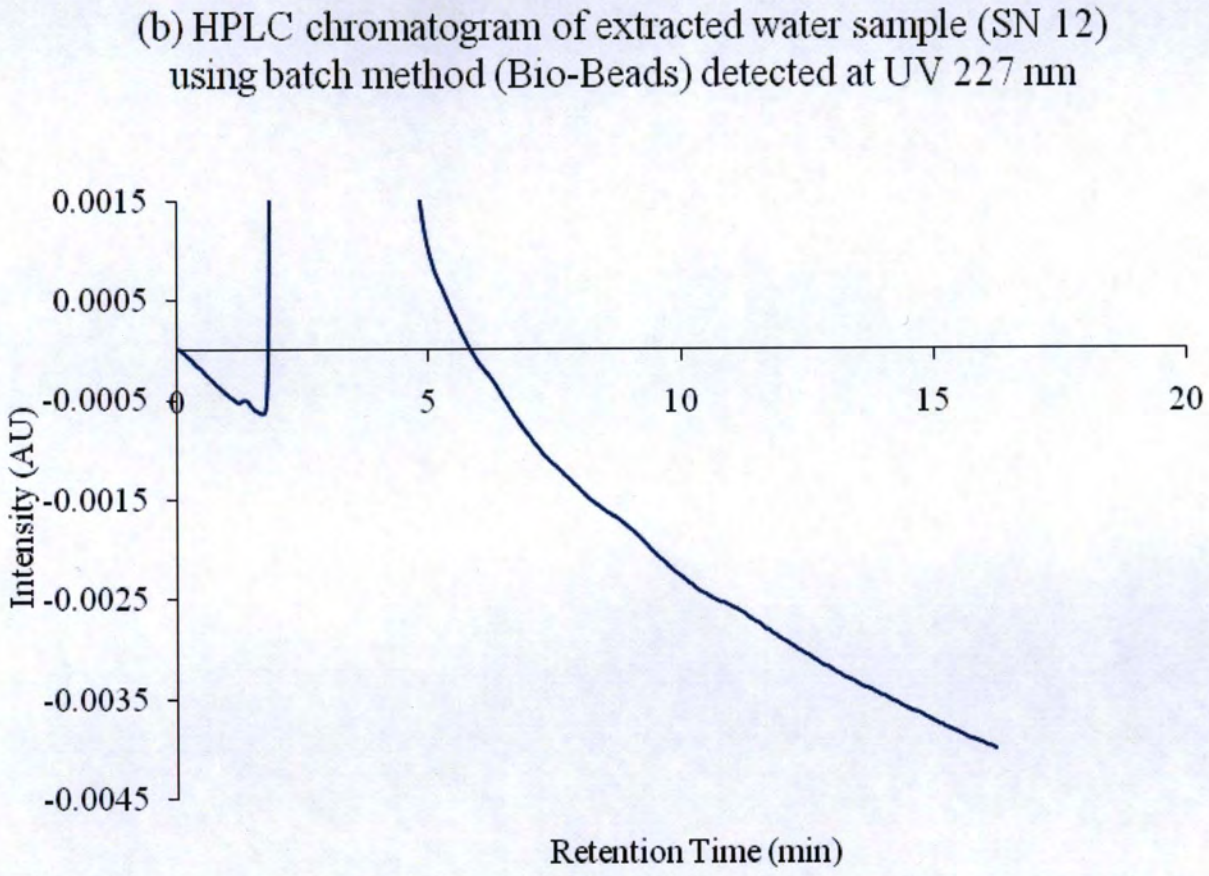


Figure 22 (b): HPLC chromatogram of extracted water sample no 12 using Bio-Beads batch method (detected at UV wavelength of 227 nm – for acesulfame-K).

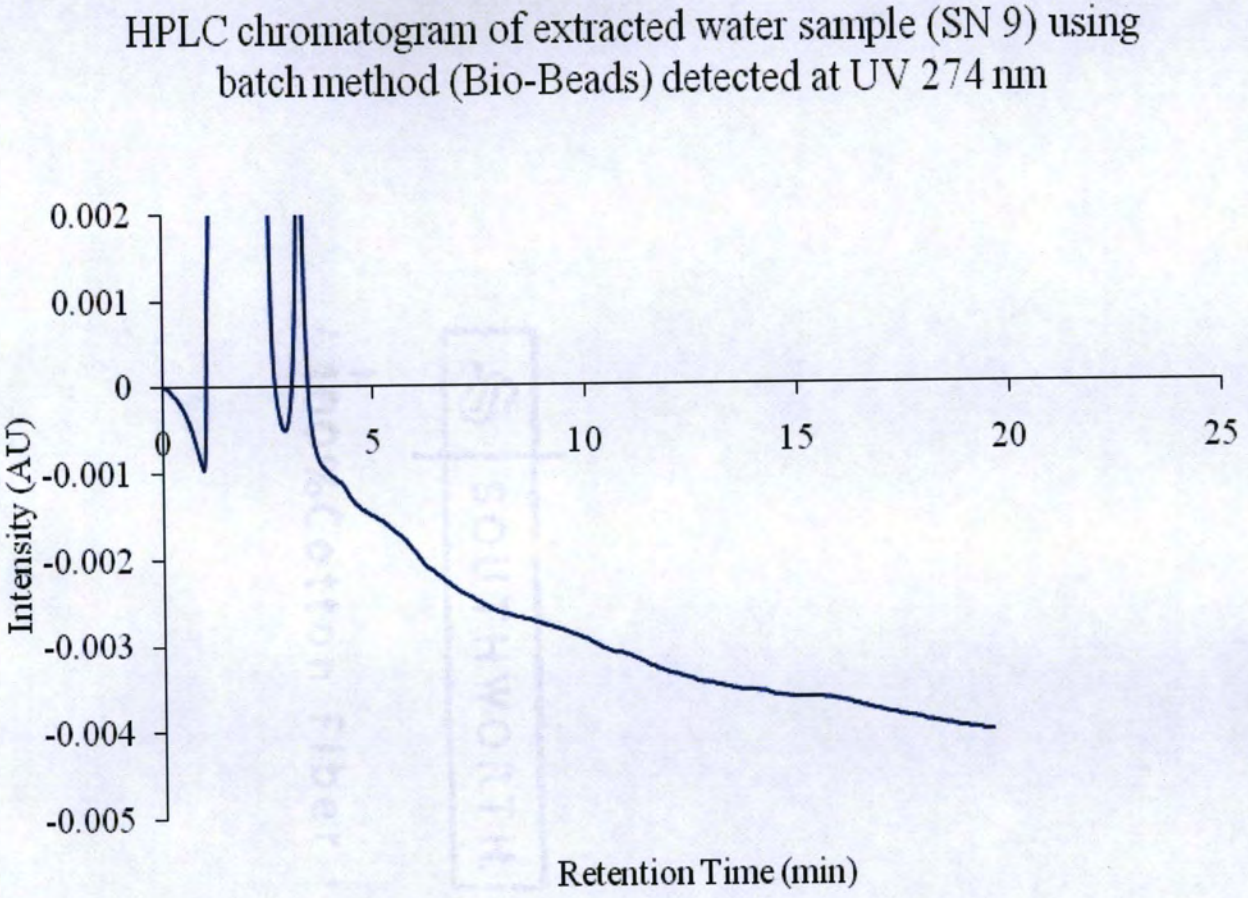


Figure 23: HPLC chromatogram of extracted water sample no 9 using Bio-Beads batch method (detected at UV wavelength of 274 nm – for caffeine).

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